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THE UNIVERSITY OF ALBERTA

LIPID MOVEMENTS IN HUMAN SKIN FIBROBLASTS

by

DONALD L. ROBERTSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING 1986

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To Dianne.

ABSTRACT

Two different types of lipid movements between serum components and cultured human skin fibroblasts were investigated. The nonreceptor mediated movement of free cholesterol between sonicated lipid vesicles and cells was studied and the effects on intracellular cholesterol metabolism were determined. The receptor mediated uptake of low density lipoprotein (LDL) -associated phosphatidylcholine (PC) and its subsequent cellular metabolism was also investigated.

In the first series of experiments, unilamellar lipid vesicles of various cholesterol to phosphatidylcholine molar ratios were used to alter, via passive exchange at the plasma membrane, the cellular free cholesterol content of cultured human skin fibroblasts which had been preincubated in lipoprotein deficient serum. The effects of these net surface transfers of cholesterol on cellular cholesterol biosynthesis, cholesterol esterification, and LDL binding were determined and were compared to the effects of cholesterol delivered to the cell interior via the receptor mediated endocytosis of LDL. Both LDL and cholesterol rich lipid vesicles increased cell cholesterol within 6 hours. Cells exposed to LDL also showed, within 6 hours, decreased cholesterol synthesis, decreased LDL binding and increased cholesterol esterification. Cells incubated with the cholesterol rich vesicles showed similar changes but these were delayed and did not occur until 24 hours. Fibroblasts incubated with cholesterol free PC vesicles had decreased cell cholesterol, increased cholesterol synthesis, increased LDL binding and decreased esterification, but only after 24 hours of incubation. Fibroblasts from a familial hypercholesterolemia (FH) homozygote, which have defective LDL receptor activity, showed qualitatively similar results after 24 hours. These results suggest that passive net movements of cholesterol occurring at the cell surface can, with time, modulate intracellular cholesterol metabolism. These findings are consistent with the idea that the movement of cholesterol from the cell surface to the cell interior is a limited and relatively slow process.

In the second series of experiments, the uptake and catabolism of various components of LDL by fibroblasts was investigated. A method was developed to prepare reconstituted LDL

particles containing exogenously added PCs. By using established procedures to radiolabel the protein and cholesteryl ester (CE) parts of the LDL molecule, it was possible to selectively follow the uptake and cellular metabolism of each of the different components. The reconstituted LDL particles were taken up specifically by the LDL-receptor pathway and delivered to the lysosomes where the CE was rapidly hydrolyzed to free cholesterol and fatty acid. The apoprotein was also degraded intracellularly to amino acids, which were released into the culture medium. The degradation of both the CE and protein was > 80% after 6 hours of incubation. Experiments with LDL which contained radiolabelled PC demonstrated that the phospholipid was taken up by normal fibroblasts but not by FH cells, which are LDL receptor deficient. However, although the LDL-associated PC was internalized in association with the protein and CE components, it appeared to escape lysosomal hydrolysis and remained substantially (> 75%) intact after 6 hours, as judged by thin layer chromatography. Incubation of cells with reconstituted LDL containing PC labelled in the choline head group resulted in the release of a small amount (5-8%) of the PC radioactivity into a water soluble form. This release was abolished in the presence of chloroquine, a lysosomal inhibitor. These results suggest that although a portion of LDL-associated PC is degraded lysosomally, a substantial fraction of the PC remains intact and appears to escape lysosomal hydrolysis. It is suggested that the lipoprotein associated phospholipid may segregate from the particle prior to arriving at the lysosome, possibly via exchange with other cellular lipids.

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Table of Contents

Chapter — Page

1. INTRODUCTION 1

1.1 General Introduction 1

1.2 The Use of Cell Culture 3

1.3 Lipid Movements Between Serum and Cells 5

1.3.1 General Introduction 5

1.3.2 Receptor Mediated Lipid Movements 6

1.3.2.1 The LDL receptor pathway 6

1.3.2.2 Other receptors which mediate lipid uptake 11

1.3.2.3 Receptor mediated efflux of lipids from cells 11

1.3.3 Non-Receptor Mediated Lipid Movements 12

1.3.3.1 General introduction 12

1.3.3.2 Cholesterol exchange 13

1.3.3.3 Net cholesterol transfer 14

1.3.3.4 Phospholipid exchange and net transfer 15

1.3.3.5 Cholesteryl ester exchange/transfer 15

1.3.3.6 Efflux of cellular lipids 16

1.3.4 Summary Of Overall Lipid Fluxes 17

1.3.4.1 Cholesterol and cholesteryl esters 17

1.3.4.2 Phospholipids 17

1.4 The Role of the Lysosome 17

1.4.1 Introduction 17

1.4.2 Lysosomal Acid Lipase 19

1.4.3 Lysosomal Phospholipases 21

1.4.3.1 Phospholipase A 24

1.4.3.2 Phospholipase C 25

1.4.3.3 Sphingomyelinase 25
1.5 Intracellular Lipid Trafficking and Metabolism 26
1.5.1 Introduction 26
1.5.2 Intracellular Lipid Movement 27
1.5.2.1 Sterol carrier protein 28
1.5.2.2 Phospholipid exchange proteins 29
1.6 Objectives 30
2. MATERIALS AND METHODS 31
2.1 Materials 31
2.2 Human Skin Fibroblasts 32
2.3 Lipoproteins 32
2.4 Lipid Vesicles 33
2.5 Cholesterol and Phosphate Determinations 33
2.6 Incubation of Cells with Lipid Vesicles 34
2.7 Acetate Incorporation into Cholesterol 34
2.8 Oleate Incorporation into Cholesteryl Esters 35
2.9 Binding of LDL 35
2.10 Preparation of Reconstituted LDL Particles 35
2.11 Incubation of Cells with Reconstituted LDL 36
2.12 Hydrolysis of ^{125}I-LDL by Cells 37
2.13 TLC of Osmium Tetroxide Treated Lipid Extracts 38
2.14 Statistical Analysis of the Data 38
3. RESULTS 39
3.1 Preparation of LDL, LPDS, and ^{125}I-LDL 39
3.2 Nonreceptor Mediated Cholesterol Movements 43
3.2.1 Cholesterol exchange between vesicles and fibroblasts 43
3.2.2 Effect of cholesterol exchange on fibroblast cholesterol content 46

3.2.3 Effect on cholesterol esterification 53

3.2.4 Effect on ^{125}I-LDL binding 55

3.2.5 Experiments with familial hypercholesterolemia fibroblasts 55

3.3 Uptake and Cellular Processing of LDL-Associated Phosphatidylcholine 60

3.3.1 Preparation and characterization of reconstituted LDL particles 61

3.3.2 Fibroblast uptake and degradation of single-labelled reconstituted LDL 65

3.3.2.1 Uptake and degradation of r[^{3}H-CE]LDL 65

3.3.2.2 Uptake and metabolism of different reconstituted LDL particles containing exogenous phosphatidylcholines 68

3.3.3 Uptake and Cellular Processing of Double-Labelled Reconstituted LDL 78

3.3.3.1 Cellular processing of rLDL labelled in PC and CE 78

3.3.3.2 Cellular processing of r[^{125}I/^{3}H-DOPC]LDL 84

3.3.3.3 Cellular uptake of rLDL containing labelled PC and cholesteryl hexadecyl ether 85

3.3.3.4 Estimation of the recycling of LDL-associated DPPC 87

3.4 Summary of the Degradation of LDL Components 93

4. DISCUSSION 95

5. REFERENCES 103

LIST OF TABLES

Table	Description	Page
1	Protein and cholesterol content of human LDL and LPDS	44
2	Lipid composition of LDL	45
3	Characterization of ^{125}I-LDL	46
4	Estimation of the amount of vesicle lipid 'sticking' to fibroblast cells	57
5	Cholesterol content of fibroblasts following incubation with media of various compositions	59
6	Effects of various incubation media on cholesterol synthesis in normal and FH fibroblast cells	69
7	Effects of various incubation media on cholesterol esterification in normal and FH fibroblasts	70
8	Chemical composition of reconstituted LDL	73
9	Phospholipid composition of r[^{3}H-DOPC]LDL	79
10	Suppression of cholesterol synthesis and stimulation of cholesterol esterification by native and reconstituted LDL	83
11	Amount of fibroblast radioactivity associated with he PC spot on tlc plates following incubation of cells with rLDLs containing radioactive PC	94
12	^{3}H/^{14}C ratio of fibroblasts following incubation with r[^{3}H-CE/^{14}C-DOPC]LDL	103
13	Quantitation of the separation of DPPC from other PC species by tlc of osmium tetroxide-treated lipid extracts	117

LIST OF FIGURES

Figure	Description	Page
1	Sequential steps in the low-density lipoprotein (LDL) receptor pathway in cultured mammalian cells	9-10
2	Pathways involved in cholesterol homeostasis in cultured fibroblast cells	19-20
3	Schematic diagram of a lysosome depicting its various degradative capabilities	22-23
4	Hydrolysis sites of the phospholipases	25-26
5	Concentration dependence of cell surface binding of ^{125}I-LDL to normal fibroblasts	48-49
6	Time dependence of binding of ^{125}I-LDL to fibroblasts at 4°C	50-51
7	Exchange of [^{3}H] cholesterol between vesicles and fibroblasts	53-54
8	Effect of albumin on [^{3}H] cholesterol exchange between lipid vesicles and normal fibroblasts	55-56
9	Effects of various incubation media on cholesterol synthesis	60-61
10	Effects of various incubation media on cholesterol esterification	64-65
11	Effects of various incubation media on LDL binding	66-67
12	Effect of phosphatidylcholine on the yield of reconstituted LDL protein	74-75
13	Effect of increasing amounts of added phosphatidylcholine on the reconstituted LDL content of the cholesteryl ester, phospholipid and [^{3}H]dioleoyl phosphatidylcholine	76-77
14	Competition curves for native or reconstituted LDL against ^{125}I-LDL binding to fibroblasts	80-81
15	Time course of the uptake and hydrolysis of r[^{3}H-CE]LDL in the presence and absence of chloroquine	84-85

16	Time course of the cellular uptake of various LDL particles reconstituted with exogenous phosphatidylcholines	87-88
17	Uptake of LDL associated DOPC by normal and FH fibroblasts	89-90
18	Radioactivity scan of a thin layer chromatogram of lipids extracted from fibroblasts incubated with r[DPPC]LDL	92-93
19	Time course of uptake of r[^{3}H-choline-DPPC]LDL by normal and FH fibroblasts	95-96
20	Time course of the appearance of ^{3}H-radioactivity in the aqueous layer of a lipid extract of normal and FH fibroblasts incubated with r[^{3}H-choline-DPPC]LDL in the presence or absence of 100 μM chloroquine	98-99
21	Time course of the cellular uptake of r[^{3}H-CE/^{14}C-DOPC]LDL	101-102
22	Time course of the hydrolysis of the CE but not the DOPC of r[^{3}H-CE/^{14}C-DOPC] LDL	104-105
23	Time course of the uptake of r[^{125}I/^{3}H-DOPC]LDL	107-108
24	Time course of the uptake and cellular hydrolysis of ^{125}I-LDL and r[^{125}I/^{3}H-DOPC] LDL	109-110
25	Concentration dependence of the uptake of r[^{3}H-CEther/ ^{14}C-DOPC]LDL by normal fibroblasts	112-113
26	Time course of the cellular uptake of r[choline-^{3}H-DPPC]LDL radioactivity into the organic and aqueous layers of a lipid extract of normal fibroblasts	115-116
27	Time course of the conversion of the DPPC or r[^{3}H-DPPC]LDL to other PC species by normal fibroblasts	118-119
28	Summary of the cellular degradation of the various components of LDL	121-122

LIST OF ABBREVIATIONS

ACAT	acyl-CoA:cholesterol transferase
apo A-1	apolipoprotein A-1
apo B	apolipoprotein B
BSA	bovine serum albumin
β-VLDL	β-very low density lipoprotein
CE	cholesteryl ester
Con A	concanavalin A
C/P	cholesterol to phospholipid molar ratio
DOPC	dioleoyl phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
EDTA	ethylenediamine tetraacetic acid
FABP	fatty acid binding protein
FCS	fetal calf serum
FH	familial hypercholesterolemia
HDL	high density lipoprotein
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-coenzyme-A reductase
^{125}I-LDL	LDL radiolabelled with ^{125}I
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein
LPDS	lipoprotein deficient serum
MEM	minimum essential medium
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine

rLDL	reconstituted low density lipoprotein
SCP	sterol carrier protein
Sil G	silica gel G
SM	sphingomyelin
TLC	thin layer chromatography

1. INTRODUCTION

1.1 General Introduction

Phospholipids and cholesterol are essential components of mammalian cells. Together they make up the lipid portion of cellular membranes which provide the compartmentalization necessary for normal cell function. They also provide a matrix for a variety of membrane bound receptors and enzymes. Cholesterol and phospholipids, particularly phosphatidylcholine, are also important structural components of the plasma lipoproteins where they act to help solubilize the more hydrophobic triglycerides and cholesteryl esters.

Although the chemical structures and biosynthetic pathways of both cholesterol and phosphatidylcholine have been known for many years, the role played by these lipids in membranes and the mechanisms by which their content within the cell is regulated remain areas of very active investigation. The precise distribution and function of cholesterol in cell membranes have not yet been established (Demel & DeKruyff, 1976; Green, 1983). There is little doubt that in mammalian cells, the phospholipid is in the relatively fluid liquid crystalline state. A variety of physical studies of both model lipid bilayers and biological membranes have demonstrated that cholesterol acts to increase viscosity and decrease membrane permeability. Hence it is generally accepted that cholesterol acts as a major modulator of the membrane physical state (Chapman, 1983; Shinitsky, 1984). There is an abundant literature that indicates that the cholesterol content of mammalian cell membranes is tightly regulated in such a way as to maintain a cholesterol to phospholipid molar ratio (C/P) compatible with optimal cellular function. Numerous studies have shown that certain membrane enzyme and receptor functions can be altered by experimental manipulations of the cellular C/P, although there remains considerable doubt as to whether the changes observed are truly physiological in nature (Sandermann, 1978).

The details of the complex biosynthesis of cholesterol from acetyl-CoA, involving over 30 enzyme catalyzed reactions, have been worked out by several groups over the last 40 years

(Bloch, 1965). Much attention has been focused on the conversion of hydroxymethyl-glutaryl-CoA to mevalonate, which is the committed step for cholesterol synthesis and which is catalyzed by the regulatory microsomal enzyme HMG-CoA reductase, which is believed to be rate limiting for the entire reaction sequence. The reaction sequence for phosphatidylcholine biosynthesis has also been established; more recently, attention has focused on the enzyme cytidylate transferase which has been shown to be rate limiting for PC synthesis in several systems (Pelech and Vance, 1984).

The regulation of cellular lipid metabolism by extracellular sources of lipid has received much attention in recent years. Cholesterol is in a state of rapid flux in most mammalian tissues with its concentration controlled via the regulation of several processes including lipoprotein transport and *de novo* synthesis. Although it had been believed for many years that liver and intestine were the only tissues in which cholesterol synthesis was subject to regulation, more recent work on cultured mammalian extrahepatic cells has demonstrated the existence of mechanisms for the control of cholesterol synthesis in peripheral tissues as well. Since cholesterol cannot be catabolized by extrahepatic tissues, their cholesterol content must therefore be determined by a balance between pathways that increase cellular cholesterol (synthesis, lipoprotein uptake) and pathways that mediate loss of sterol from the cell to the plasma, presumably for return to the liver (reverse cholesterol transport).

The regulation of cell phosphatidylcholine content remains even less well understood. Although some of the factors influencing the rates of PC biosynthesis are now known, the effects of movements of extracellular phospholipid into the cell and the influence of the rates of phospholipid degradation on the overall picture remain to be elucidated. There is also some evidence for coordination of sterol and phospholipid synthesis in cultured cells (Cornell and Horwitz, 1980), but in general the influences of different cellular lipids on each others' metabolism have not yet been investigated.

The study of the factors which regulate the balance of cholesterol and phospholipid between cellular and extracellular compartments has considerable clinical relevance as well.

Repeated epidemiologic studies have demonstrated the positive relationship between the level of serum cholesterol and the risk of coronary heart disease (Kannel *et al.*, 1973) and much attention has been directed at reducing atherosclerosis through alterations in plasma lipids (Lipid Research Program, 1984; Schaefer and Levy, 1985; Blankenhorn, 1985). Exactly how plasma lipoproteins contribute to atherogenesis is not known. Ross and Glomset (1973) suggested that chronic hyperlipidemia may contribute to the development of atherosclerosis by direct damage to the endothelial lining of the arterial wall. Another possible mechanism is the transfer of lipoprotein lipid to cell membranes, thereby producing alterations in their normal physiological properties, perhaps including enhanced proliferation (Gotto & Jackson, 1978; Papahadjopolous, 1974).

1.2 The Use of Cell Culture

The development of methodology which permitted the establishment and prolonged cultivation of single cell types in media of defined composition brought about a new era in the study of the regulation of mammalian lipid metabolism. Cell culture provided the opportunity for much greater control of experimental conditions than in the tissue slice or whole animal. Individual cell types could be studied independent of the complicating tissue or organ influences. It was possible to manipulate the composition of the growth media and to study the movements of various lipid components between the cellular and extracellular compartments.

The pioneering studies of Bailey (Bailey, 1966, 1967), Rothblat (Rothblat and Kritchevsky, 1967; Rothblat, 1972) and Geyer (Geyer *et al.*, 1961; Geyer, 1967) demonstrated that cells grown in culture medium supplemented with serum derive much of their lipids from that serum. When the serum was not included in the culture medium, the cells responded by synthesizing the required phospholipids, triglycerides, and cholesterol from various water soluble precursors, primarily acetate and glucose. Together these two observations implied that certain serum components could suppress cellular lipid biosynthesis and that there were feedback controls regulating *de novo* synthetic pathways in response to changes in the lipid

content of the growth medium.

The use of cell culture has also allowed the investigation in a controlled environment of metabolic changes resulting from deficiencies of either cellular enzymes or membrane receptors. Mutant cells can be compared to their normal counterparts, and this has proven to be very useful in the elucidation of many underlying biochemical processes. Human skin fibroblasts, which are relatively easy to obtain and grow, are now extensively used in the diagnosis and study of metabolic disorders.

It was the study of cholesterol metabolism in the cultured cells of patients with one such disorder, familial hypercholesterolemia (FH), which led Goldstein and Brown (1973) to the discovery that the uptake of low density lipoproteins (LDL) could be mediated by a specific cell membrane receptor. This concept has been elaborated into the 'LDL receptor hypothesis' for the regulation of cellular free cholesterol content (Goldstein and Brown, 1977; Goldstein and Brown, 1983). The receptor mediated endocytosis of LDL and its role in cholesterol homoeostasis will be discussed in more detail below.

The use of human cells grown in culture has also been essential in the characterization of a large number of lysosomal storage diseases, genetic disorders in which one or more lysosomal enzymes are lacking. Several of these disorders involve the absence of lysosomal hydrolases which participate in lipid metabolism, and these will be discussed below.

Despite the considerable advantages provided by cell culture, it is important to recognize that the method is not absolute. It must be kept in mind that culture conditions are artificial and subject to potential artefacts including silent infections, pH changes, freedom from hormonal influence, and potential nutritional deficiencies. Cells maintained in culture will likely undergo different selection pressures depending on the particular growth environment provided. The cell density achieved is usually much lower than *in vivo*, and the concentrations of nutrients, lipoproteins, and growth factors provided is undoubtedly different. This may be particularly relevant when studying the lipid metabolism of groups of cells grown in medium containing different preparations of serum. Culture of single cell types also has the

disadvantage that cell-cell interactions are eliminated. There is evidence, for example (Quinn *et al.*, 1985), that arterial endothelial cells may have important modifying effects on the composition and concentration of lipoproteins which interact with macrophages and smooth muscle cells.

Despite these limitations, the study of lipid movements between serum and cells grown in tissue culture has provided many insights into cellular metabolism of lipids and their involvement in pathological conditions.

1.3 Lipid Movements Between Serum and Cells

1.3.1 General Introduction

There is a dynamic interchange of lipids between lipoproteins and biological membranes. Flux of various lipids between different types of synthetic lipid vesicles, lipoproteins, cells, and cell organelles have been extensively studied (Bell, 1977, 1978; Spector *et al.*, 1981; Green, 1983). It has become apparent that for mammals, the overall flux of lipid between the plasma and various tissues is the result of a very complex and dynamic set of interactions between the different lipoprotein classes (which undergo constant metabolism, frequently involving bidirectional transfers of constituents) and the plasma membranes of the wide variety of cell types present in the body. These interactions may involve specific, high affinity receptors on the cell surface -- in this case the movement of lipid between plasma and the cell is directed by the presence of the appropriate apoprotein. Also important are transfers of different lipid classes between lipoproteins and cells which occur by physicochemical mechanisms. In this case the flux of the particular lipid class will depend partly on its physical properties and also in some way on the properties of the donor and acceptor membranes. These kinds of transfers of lipids do not require energy, but may be catalyzed by a variety of different exchange proteins present either in plasma or within the cell cytosol.

Determining the relative contributions that these receptor mediated and non-receptor mediated movements make to the overall flux of lipid between the extracellular (plasma) and cellular compartments *in vivo* has been a challenging task. In the effort to simplify a very complex set of dynamic interactions, many workers have studied lipid movements in model systems, with relatively simple cells such as erythrocytes, or with cells grown in tissue culture.

Cells grown in tissue culture can derive lipid via either *de novo* biosynthesis or uptake from exogenous sources in the medium. Serum forms the major lipid source for cultured cells. Phospholipids, triacylglycerols, cholesterol, and cholesteryl esters are contained in serum lipoproteins and it has been well established that many cultured cells will utilize these lipids when they are added as components of serum or as isolated plasma lipoprotein fractions (Bailey, 1967; Rothblat, 1972). Since then, many studies have been directed at determining the precise mechanisms by which various types of lipids are taken up, transported within, and utilized by cultured cells, and the effects that these processes have on cellular lipid metabolism.

1.3.2 Receptor Mediated Lipid Movements

1.3.2.1 The LDL receptor pathway

It has been appreciated for many years that hepatic cholesterol biosynthesis is under negative feedback control by cholesterol in the diet (Gibbons *et al.*, 1982), and that the site of dietary feedback control is HMG CoA reductase. *In vivo* studies implicated the liver and the intestine as the principal organs of cholesterol synthesis and it was generally presumed that these were the only sites which had regulation of cholesterol metabolism. It was not until the advent of cell culture techniques that the feedback control of cholesterol biosynthesis was demonstrated in other cell types from peripheral tissues as well. In a variety of studies it was demonstrated that cellular cholesterol synthesis was reduced when whole serum was included in the culture medium. (Bailey, 1966; Avigan *et al.*, 1970, 1972; Williams and Avigan, 1972).

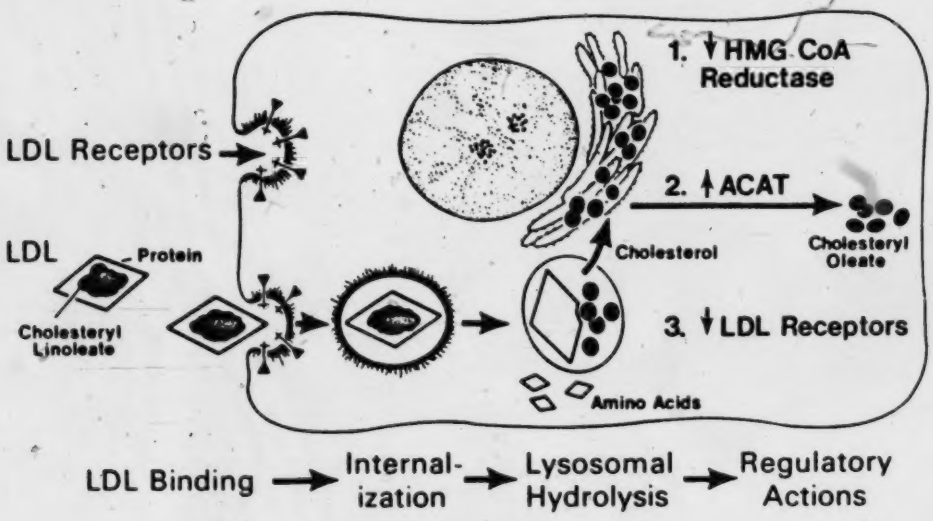
It was against this background that Brown and Goldstein began their elegant series of experiments which eventually led to the demonstration of a specific membrane receptor which could mediate the interaction of LDL with human fibroblasts in culture. Their first important observation (Brown *et al.*, 1973) was that removal of serum from the medium resulted in a large increase in HMG CoA reductase activity. This increase could be reversed by whole serum, by LDL at low concentrations, or by HDL at very high concentrations. This reversal could not be achieved by the addition of lipoprotein deficient serum (LPDS), which is serum from which the lipoproteins have been removed by ultracentrifugation. It was suggested that cholesterol biosynthesis in extrahepatic tissues is constantly suppressed via negative feedback control of HMG-CoA reductase by LDL (Brown *et al.*, 1973). Further contemporary studies showed that this feedback control was achieved by changes in the rate of enzyme synthesis, rather than by changes in degradation or via some kind of direct mechanism (Brown *et al.*, 1973*b*; Higgins and Rudney, 1973; Edwards and Gould, 1972). More recent investigations have also implicated a possible role for enzyme phosphorylation/dephosphorylation in the regulation of HMG-CoA reductase (Scallen and Sanghvi, 1983). There is also evidence that the physical state of the endoplasmic reticulum lipids influence the reductase activity (bin Sipat and Sabine, 1981; Venkatesan and Mitropoulos, 1982).

The search for the molecular mechanism by which LDL exerted its suppressive effects on cellular cholesterol biosynthesis was greatly aided by the study of a human genetic disorder called familial hypercholesterolemia (FH). FH is a rare, autosomal codominantly inherited disease characterized clinically by markedly elevated serum LDL cholesterol (240 mg/dl for heterozygotes, 625 mg/dl for homozygotes) and premature, accelerated atherosclerosis (Goldstein and Brown, 1983). Study of cultured fibroblasts from FH patients revealed that they lacked the LDL-mediated down regulation of HMG CoA reductase, even though the kinetic properties of the enzyme appeared to be normal (Brown *et al.*, 1974a; Goldstein and Brown, 1973). It was suggested that the genetic defect

in FH was somehow impairing the delivery of LDL cholesterol into the cell. This was confirmed by studies of the interaction of ^{125}I-radiolabelled LDL with FH cells which showed both defective binding (Brown & Goldstein, 1974a; Anderson *et al.*, 1976) and degradation (Goldstein & Brown, 1974) of the LDL protein. Since only the high affinity, saturable component of total LDL binding and degradation was defective in the FH cells, and since this high affinity component could be removed from normal fibroblasts by pretreatment of the cells with the proteolytic enzyme pronase, Goldstein and Brown (1974) postulated the existence of a specific cell surface receptor for LDL. This concept of delivery of LDL lipid to cells by a receptor mediated endocytic pathway has been elaborated into a hypothesis for the regulation of cellular free cholesterol content (Brown and Goldstein, 1979; Goldstein and Brown, 1977) (Figure 1). In this model, the cholesteryl esters, free cholesterol and phospholipids of LDL are delivered to the cell surface via high affinity cell surface receptors which recognize the protein component of LDL, apolipoprotein B. The LDL particle is internalized into the cell by endocytosis and delivered to the lysosomes, where the apoliprotein is degraded to its component amino acids and the cholesteryl esters are hydrolyzed by an acid hydrolase, yielding free cholesterol and free fatty acids. The cholesterol so liberated can be used for membrane synthesis or re-esterified into the cholesteryl ester form within the endoplasmic reticulum. This process of internalization and degradation of LDL has several regulatory consequences:

1. a suppression of *de novo* cholesterol biosynthesis, achieved through a reduction in the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme-A reductase, the rate limiting enzyme in cholesterol biosynthesis,
2. a stimulation of cholesterol esterification, achieved through an increase in the activity of the microsomal enzyme acyl-CoA: cholesterol transferase,
3. a decrease in the synthesis and thus the number of LDL receptors.

Figure 1. Sequential steps in the low-density lipoprotein (LDL) receptor pathway in cultured mammalian cells. (From Goldstein *et al*, 1983).



Apparently, therefore, cultured cells have their cholesterol content regulated through a complex feedback mechanism which responds to cholesterol delivered via the LDL receptor pathway. Under normal conditions, cells obtain much of the cholesterol required for membrane synthesis via the internalization and degradation of LDL. Endogenous cholesterol synthesis is greatly reduced, esterification of any excess cholesterol delivered by LDL is enhanced, and the cells adjust the number of LDL receptors to a basal level that provides for the entry of only as much LDL cholesterol as is required.

The elucidation by Goldstein and Brown and coworkers of these mechanisms for the regulation of cell cholesterol content was a brilliant demonstration of the analytical potential of cell culture and also contributed significantly to the development of the paradigm of receptor mediated endocytosis (Goldstein *et al.*, 1979). However, the significance of the LDL receptor mediated pathway to the organism as a whole is far from clear. In general, rates of whole body cholesterol synthesis in FH homozygotes are similar to that of normal controls (Fielding and Fielding, 1985). Also, in tissue culture systems, it appears that it is sterol depletion of the cells, rather than an absence of LDL in the culture medium, which is the predominant signal for the induction of new LDL receptor synthesis (Oram *et al.*, 1980; Fielding and Fielding, 1985). These types of observations strongly imply that the LDL receptor pathway is not necessarily rate limiting for cellular cholesterol synthesis rates, either for cells grown in the presence of LDL in culture, or *in vivo* (Stange & Dietschy, 1983). In light of the above, more attention has been focused on the roles played by lipid exchange processes in determining cellular lipid metabolism and content.

Although it appears that the receptor mediated uptake and degradation of LDL is not rate limiting for cholesterol synthesis *in vivo*, clearly much LDL is degraded by receptor mediated processes. In terms of the lipid components of the particle, this represents a flux into the cells of cholesteryl esters, free cholesterol, and also phospholipid. This pathway may have particular significance for the movement of phospholipids into cells. The LDL particle is greater than 20% phospholipid by weight, primarily phosphatidylcholine (PC)

and sphingomyelin (SM) (Lee, 1976). The rate of non-receptor mediated movements of these phospholipids from the plasma to cells has not been unequivocally determined, but it is clear that it will be much less than that of free cholesterol, which has a much higher critical micellar concentration and is therefore more likely to move via a diffusional mechanism. Thus far very few studies have been directed at examining the uptake and metabolism of lipoprotein associated phospholipid, or determining the effects this uptake may have on endogenous lipid metabolism.

1.3.2.2 Other receptors which mediate lipid uptake

In addition to the so-called LDL receptor (which recognizes apolipoproteins B and E), several other receptors have been described which may participate in the delivery of lipids to cells (Brown and Goldstein, 1983; Mahley and Innerarity, 1983). These are:

1. The scavenger receptor of macrophages and endothelial cells, which recognizes chemically modified LDL and which may play a role in the development of the "foam cells" of the atherosclerotic plaque,
2. The β-VLDL receptor, which is also present on macrophages and endothelial cells and which recognizes a minor, cholesterol rich subfraction of VLDL,
3. The apo E receptor, which is a hepatic receptor involved in the clearance of chylomicrons and possibly VLDL remnants.

Once again, the physiological significance of these receptors *in vivo* is not entirely clear and is a current area of active investigation.

1.3.2.3 Receptor mediated efflux of lipids from cells

This discovery of the LDL receptor also stimulated the search for other cell surface receptors that would recognize apolipoproteins other than the apo B of LDL. In particular, much attention has been focused on receptors that recognize the apolipoproteins of high density lipoprotein (HDL). It has been proposed (Glomset, 1968) that HDL may remove cholesterol from peripheral cells, possibly via some kind of surface transfer process, and

return it to the liver for disposal; this has been called "reverse cholesterol transport". This hypothesis has been intensively investigated (for a review see Tall and Small, 1980). Various studies have demonstrated that HDL is not catabolized via the LDL receptor pathway (Miller *et al.*, 1977; Stein *et al.*, 1976; Daerr *et al.*, 1980). It has been shown that cellular cholesterol efflux results in increases in sterol synthesis and LDL binding, and decreases in esterification, but the identity of the physiological cholesterol acceptor, the role of the apolipoproteins, and mechanisms by which cholesterol depletion at the plasma membrane influences intracellular metabolism are all yet unclear.

There is now good evidence that in the rabbit, HDL is the physiological mediator of reverse cholesterol transport *in vivo* (Miller *et al.*, 1985). Although the search for an HDL receptor has been hampered in the past by the apparent cross reactivity of HDL for the apo B, E (LDL) receptor, more recently progress has been made. A high affinity receptor that recognizes HDL has been demonstrated in human skin fibroblasts and cultured arterial smooth muscle cells (Biesbroek *et al.*, 1983). The activity of this receptor is upregulated by cholesterol loading (Oram *et al.*, 1983), and it has been postulated that this receptor may play a role in reverse cholesterol transport from peripheral cells (Oram, 1983). Recently, a saturable receptor specific for HDL has also been shown to be present on human liver membranes (Hoeg *et al.*, 1985). This receptor activity is upregulated by cholesterol loading, and it is possible that it may play some role at the delivery end of the proposed HDL reverse cholesterol transport mechanism.

1.3.3 Non-Receptor Mediated Lipid Movements

1.3.3.1 General introduction

A spontaneous, protein independent transfer of lipid molecules will occur between a wide variety of membranes. Lipid exchange is the one-for-one exchange of identical molecules. Net transfer may occur when the movement is unidirectional, or more likely, when bidirectional but occurring at different rates. Lipid exchange/transfer is a

physicochemical process and does not require metabolic energy. Lipid exchange processes play an important role in determining the overall fluxes of various lipid components, both *in vivo* and in the tissue culture flask. The composition of the maturing, circulating plasma lipoproteins is constantly changing, partly due to catabolism but due also in large measure to the net transfer of lipids between each other and with cells. The magnitude and direction of these net transfers depends on the physical nature of the donor and acceptor membranes (for example, the C/P ratio), and on the physical properties of the lipid itself. In general, cholesterol and fatty acids move rapidly between membranes or lipoproteins, phospholipids move more slowly, and cholesteryl esters move very slowly (discussed in more detail below). All of these lipid components have the potential to be cleared from the plasma *(in vivo)* or from the culture medium *(in vitro)* as single molecules, by moving down their chemical potential gradients. These non-receptor mediated movements of lipids between serum and cells do not depend on macromolecular uptake of intact lipoproteins, but it should be remembered that these processes occur simultaneously with intact uptake via nonspecific pinocytosis or adsorptive endocytosis, as well as via receptor mediated endocytosis. However, it is becoming increasingly apparent that non-receptor mediated uptake of lipid from plasma or serum may form a significant part of the total lipid flux under physiological conditions.

1.3.3.2 Cholesterol exchange

Cholesterol exchange is a general phenomenon that occurs wherever cholesterol is present, either *in vivo* or *in vitro*. Free cholesterol has been shown to move between a variety of biological and model lipid membranes via a passive exchange process. In many instances this movement is a simple one-for-one exchange that does not change the cholesterol content of donor or acceptor membranes. There is now considerable evidence suggesting that the mechanism of cholesterol exchange involves the partitioning of cholesterol out of the membrane bilayer into the aqueous phase through which it then diffuses (McLean and Phillips, 1981; Backer and Dawidowicz, 1981; Rothblat and Phillips, 1982);

for a contrary view see Jonas and Maine (1979). There is a huge literature on the exchange of radioactive cholesterol in a wide variety of systems (for reviews see Bell, 1978; Green, 1983). Cholesterol exchange will occur between lipid vesicles and human skin fibroblasts (Poznansky and Czekanski, 1982) or vesicles and rat arterial smooth muscle cells (Slotte and Lundberg, 1983*a*). It has also been demonstrated that the free cholesterol of LDL will exchange with the cellular cholesterol of fibroblasts (Shireman and Remsen, 1982; Goldstein *et al.*, 1979) and adipocytes (Angel *et al.*, 1981).

1.3.3.3 Net cholesterol transfer

Net movements of cholesterol between model lipid membranes, lipoproteins, red and white blood cells, and cultured cells can also occur via passive exchange. The accumulation of cellular cholesterol resulting from net transfer from lipoproteins has been extensively studied in red cells (Cooper, 1978). Model lipid donors and acceptors have also been utilized extensively to study the net transfer process. It has been observed in many different systems that lipid vesicles with relatively high C/P ratios (> 1) will transfer cholesterol to cells, while with a ratio of unity little or no net transfer occurs (Arbogast *et al.*, 1976). It can therefore be concluded that for relatively simple systems, such as lipid vesicles or erythrocytes, the overall cholesterol flux occurs by a net transfer process, the driving force for which appears to be related to the difference in the C/P ratios of the donor and acceptor membranes (Lange and D'Alessandro, 1977).

For more physiological systems, involving metabolically active cells cultured in serum-containing medium, the situation is complicated by any receptor mediated pathways (particularly the LDL receptor pathway) and by potential sterol fluxes occurring via net transfer to or from other serum components. The contribution of these kinds of non-receptor mediated surface transfer movements of cholesterol to the overall sterol flux between serum lipoproteins remains unclear, but various studies have suggested that LDL cholesterol may move independently of the lipoprotein particle both *in vivo* (Shireman and Remsen, 1982) and *in vitro* (Portman *et al*, 1980). It has also been suggested that in

atherosclerosis, where there is a progressive accumulation of cellular lipids (particularly cholesterol and cholesteryl esters) the surface transfer of lipids may represent a highly significant route of lipid entry (Bell, 1978).

1.3.3.4 Phospholipid exchange and net transfer

The exchange of various phospholipids has been demonstrated among lipoproteins (Minari and Zilversmit, 1963), between plasma and erythrocytes (Hagerman and Gould, 1951; Reed, 1968), and between a variety of intracellular organelles (Wirtz and Zilversmit, 1968, 1969; McMurray and Dawson, 1969). In addition, a variety of cytosolic proteins capable of catalyzing phospholipid exchange have been isolated and characterized (Wirtz, 1974; Zilversmit and Hughes, 1976). Studies of phospholipid exchange in model lipid vesicle systems suggest that transfer occurs via soluble lipid monomers (Roseman and Thompson, 1980; Nichols and Pagano, 1981).

There has been very little work done on establishing the role of extracellular sources of phospholipid in the regulation of intracellular phospholipid content, composition, synthesis, or degradation. Recently a plasma phospholipid transfer protein has been isolated (Tall *et al.*, 1983) but its physiological role in promoting movement of phospholipids between lipoproteins and cells is not known.

1.3.3.5 Cholesteryl ester exchange/transfer

It has been demonstrated in several cell types that there is some transfer of cholesteryl esters from triacylglycerol rich lipoproteins across the plasma membrane into the cell interior (Fielding and Fielding, 1985). Although this process has the characteristics of a receptor mediated endocytic pathway, it does not appear to involve uptake of the intact lipoprotein. How this transfer is achieved is not known, but it is known that the process is stimulated by the action of lipoprotein lipase, and it may be that other products of this enzyme (fatty acids, lysophospholipids, or monoglycerides) promote the uptake of cholesteryl ester by locally modifying membrane bilayer stability.

1.3.3.6 Efflux of cellular lipids

One of the possible ways in which serum components could interact with cells is to promote the removal of cellular lipids, especially cholesterol. Many authors have observed efflux of cholesterol from cells grown in culture. In many cases the actual efflux measured has been the efflux of radioactivity from cells which had been prelabelled by either the addition of radioactive cholesterol in the medium (Burns and Rothblat, 1969) or prelabeled biosynthetically from radioactive acetate or mevalonate (Poznansky and Czekanski, 1982).

Many investigations have been made of the ability of lipid vesicles, specific lipoprotein classes, and apoprotein lipid recombinants to promote efflux of cholesterol from cultured cells (Stein *et al.*, 1975, 1978; Burns and Rothblat, 1969; Bates and Rothblat, 1974; Bellini *et al.*, 1984). Despite much work in this area, identification of a physiological cholesterol acceptor in a tissue culture system has proven to be difficult. Many attempts to measure cholesterol efflux from cultured cells were based on the quantitation of isotopically labelled sterol, and under these conditions it is impossible to distinguish net movement of cholesterol from dilution of radiolabel by exchange. Studies of net cholesterol efflux from cultured cells to HDL or HDL-like particles have in general shown that only a limited amount of cell plasma membrane cholesterol can be removed (Stein and Stein, 1973; Stein *et al.*, 1976; Phillips *et al.*, 1980; Daniels *et al.*, 1981).

There is, however, substantial evidence that the reverse cholesterol transport from peripheral cells into the circulation can be driven by the esterification of cholesterol to cholesteryl ester by plasma lecithin cholesterol acyl transferase (LCAT) (Fielding and Fielding, 1985). It appears that the physiological lipoprotein acceptor is a rapidly turning over subfraction of HDL particles which contain only apo A-1, and that this species is the preferred substrate for LCAT. This process of cholesterol esterification serves to maintain a negative free cholesterol gradient between the extracellular fluid and the cell membrane (Fielding and Fielding, 1982). The influence of the recently described HDL receptors (see section 1.3.2.3) on this process is not known.

1.3.4 Summary Of Overall Lipid Fluxes

1.3.4.1 Cholesterol and cholesteryl esters

The pathways which are involved in cellular cholesterol homeostasis are outlined in Figure 2. Several of these pathways promote increased cell cholesterol content:

1. receptor mediated uptake of intact lipoproteins (for example, the LDL receptor pathway),
2. cellular cholesterol biosynthesis, regulated at the level of HMG CoA reductase,
3. net cholesterol uptake by lipid transfer from cholesterol rich lipoproteins,
4. hydrolysis of cellular cholesteryl ester stores by a cytoplasmic neutral esterase (Brown *et al.*, 1980).

There are only two known pathways by which cells in culture or *in vivo* (with the exception of the liver and adrenals) can decrease their cholesterol content:

1. esterification of cholesterol, catalyzed by ACAT,
2. net cholesterol efflux to extracellular acceptors, particularly HDL.

1.3.4.2 Phospholipids

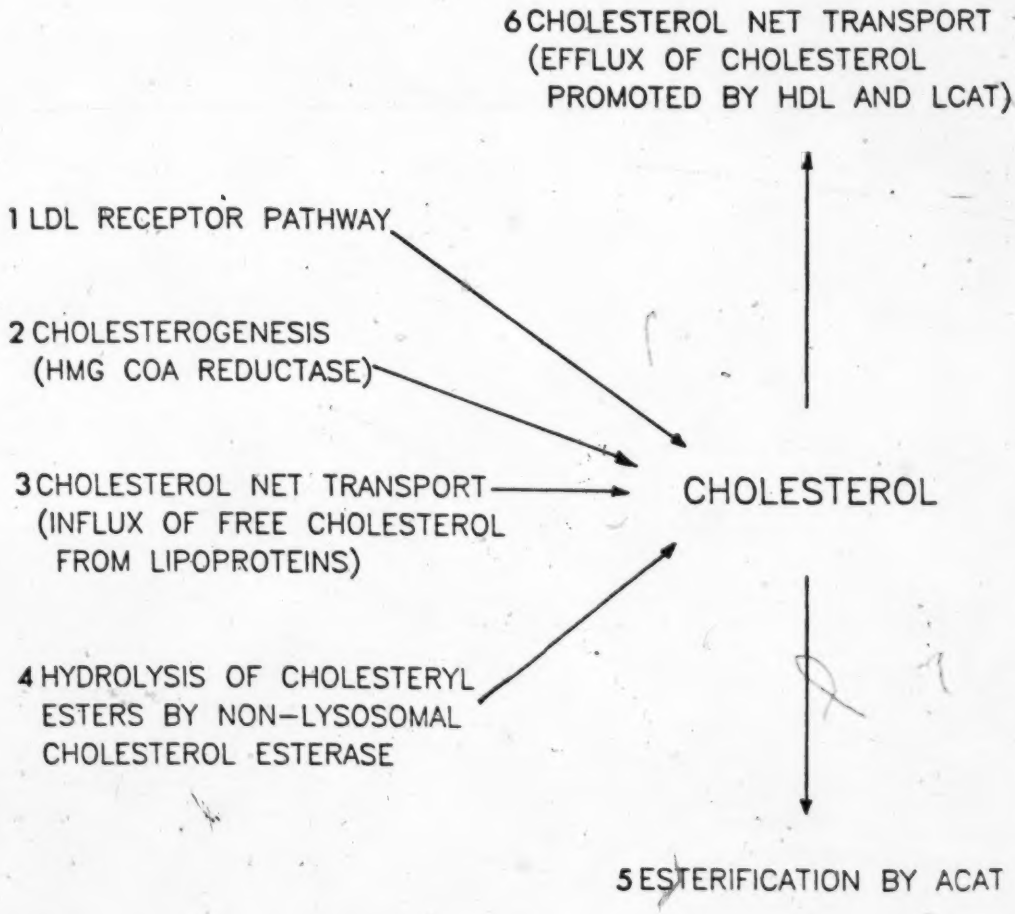
In general, the flux of phospholipids between cells and either serum or plasma and the influence that this has on the regulation of cellular phospholipid content and composition are still not well understood.

1.4 The Role of the Lysosome

1.4.1 Introduction

Turnover of cellular components has been recognized for many years but it was not until the demonstration by De Duve *et al.* (1955) of the latency of acid hydrolases in liver homogenates that it was realized that degradation was compartmentalized in cellular organelles that came to be known as lysosomes. Lysosomes have been shown to contain a variety of

Figure 2. Pathways involved in cholesterol homeostasis in cultured fibroblast cells.



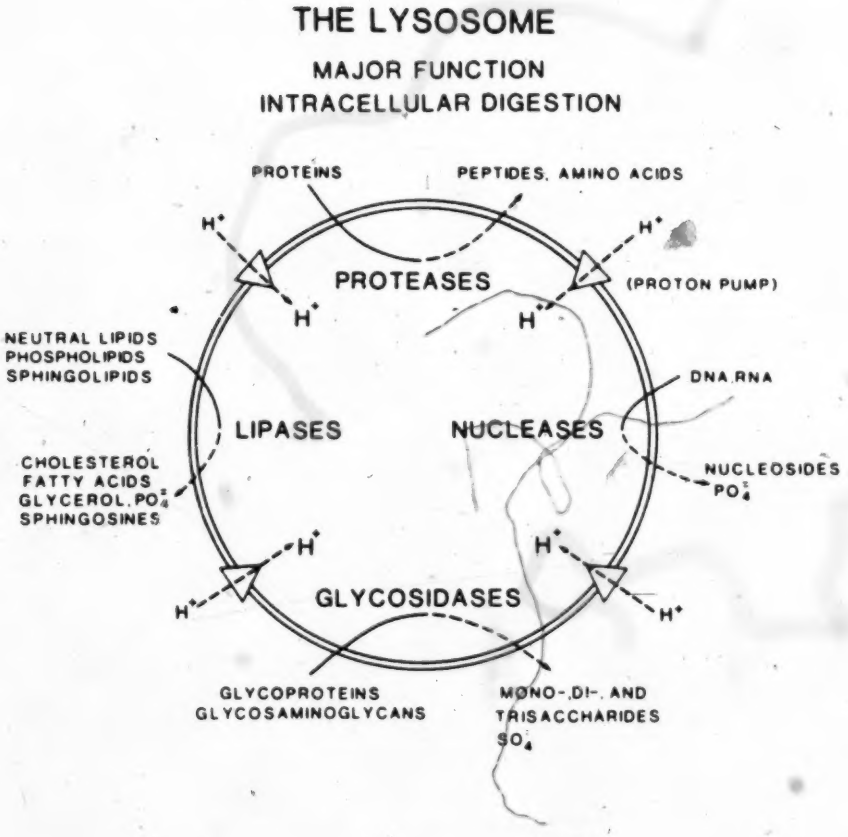
degradative enzymes (Figure 3). Human pathology has clearly indicated the essential role of the lysosome in metabolic turnover, as a variety of inherited deficiencies of acid hydrolases have been characterized and in each case the corresponding substrates continue to be synthesized but are not properly degraded and accumulate instead in enlarged lysosomal bodies (Dingle, 1977).

It is clear that lysosomes participate in the cellular degradation of neutral lipids, phospholipids, and sphingolipids (Barrett and Heath, 1977). A number of genetic disorders arising from deficiences in acid lipase (Fowler and Brown, 1984) or sphingomyelinase (Brady, 1983) have been described. Although there are no known genetic mutations of lysosomal phospholipases, treatment of animals or humans with a variety of phospholipase inhibitors has been shown to result in lipid storage disorders, with lysosomal phospholipid accumulation (Matsuzawa *et al.*, 1977; Matsuzawa and Hostetler, 1980; Blackwell and Flower, 1983; Hostetler *et al.*, 1985). It has only been relatively recently that the various lipase activities present in lysosomes have been characterized, and as yet there have been very few evaluations of the role of these enzymes in overall cellular lipid metabolism. Lysosomal lipases participate in the degradation of material internalized into the cell either by phagocytosis or receptor mediated endocytosis (Silverstein *et al*, 1977). Although there is considerable morphological evidence for autophagy, the role of the lysosomal lipases in the turnover of endogenous lipid has yet to be elucidated.

1.4.2 Lysosomal Acid Lipase

It has become apparent in the last few years that the hydrolysis of neutral lipids in lysosomes occurs as a result of the action of a single enzyme, acid lipase. A wide variety of different lipase activities have been described (reviewed by Fowler and Brown, 1984) but it is likely that the substantial differences in pH optima, substrate specificity, and effects of cofactors reported are a result of the wide variety of enzyme preparations and assay procedures used, rather than due to the existence of multiple enzyme activities.

Figure 3. Schematic diagram of a lysosome depicting its various degradative capabilities (From Fowler and Brown, 1984).



Purification of acid lipase has been retarded by its extreme hydrophobicity and instability. A triacylglycerol lipase, which cleaves the fatty acyl moieties from triacylglycerols, was purified from rat liver by Teng and Kaplan (1974). It was reported to have substantial cholesterol esterase activity, but did not hydrolyze phospholipids. Cholesterol esterase (cholesterol ester hydrolase) was shown to be present in macrophage lysosomes (Werb and Cohn, 1972). *In vitro* its activity is dependent on the presence of phospholipid and detergent, and the reaction can be reversed if the appropriate concentrations of cholesterol, cholesteryl esters, and phospholipids are present (Nilsson, 1973). It has also been purified from rat liver lysosomes by Brown and Sgoutas (1980), from rabbit by Imanaka et al. (1981), and from human liver by Warner *et al.* (1981). In all three cases simultaneous purification of lipase activity against trioleoylglycerol and cholesteryl oleate was obtained, strongly implying that lysosomal triacylglyerols and cholesteryl esters are hydrolyzed by the same enzyme.

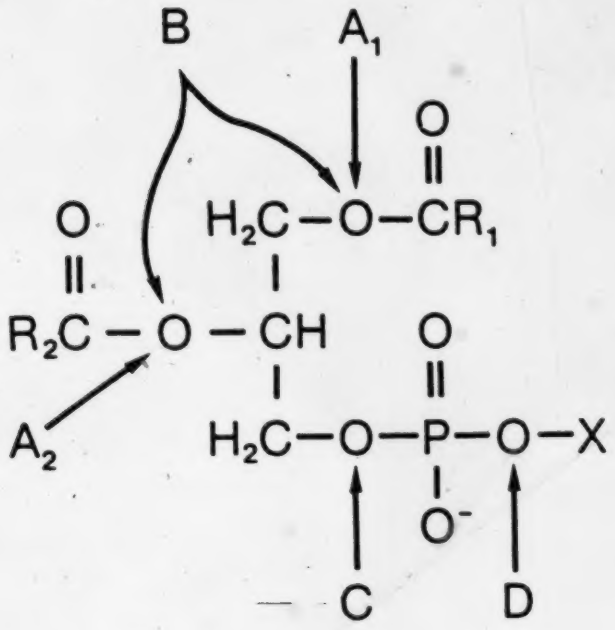
Cholesterol esterase activity has also been studied in human skin fibroblasts from both normal subjects and subjects with cholesteryl ester storage disease (Goldstein *et al.*, 1975). Cells from patients with this disorder have deficient lysosomal acid lipase activity, and accumulate triacylglycerols and especially cholesteryl esters. These studies have demonstrated the essential role of lysosomal cholesterol esterase activity in the hydrolysis of LDL-associated cholesteryl esters with subsequent regulation of intracellular cholesterol metabolism (Krieger *et al.*, 1978; Brown *et al.*, 1976).

Lysosomes have also been shown to contain various phospholipases as well as a sphingomyelinase. These activities are considered in more detail below.

1.4.3 Lysosomal Phospholipases

Phospholipases are widely distributed enzymes that catalyze the hydrolysis of phospholipids. They are classified by their site of attack on the phospholipid molecules (Figure 4). Phospholipase A_1 and phospholipase A_2 are acyl hydrolases, and catalyze the hydrolysis of the 1 or 2-acyl ester bond to yield a lysophospholipid and a free fatty acid. There are some

Figure 4. Hydrolysis sites of the phospholipases.



phospholipases which can hydrolyze both acyl chains (phospholipases B) as well as a variety of enzymes which attack monoacyl phospholipids (lysophospholipases). Phospholipase C and phospholipase D are phosphodiesterases and catalyze cleavage of either the glycerol-phosphate or phosphate-base bonds.

Phospholipases can be separated into two broad categories based on their location, function, and physical properties. The best characterized are the extracellular phospholipases which are found in bacterial toxins, various venoms, and digestive juices. In general these enzymes have a completely digestive function and do not appear to be regulated.

The other group of phospholipases are the intracellular phospholipases. These have been described in a wide variety of tissue and cell types and in a large number of subcellular locations (van den Bosch, 1980). Many of these enzymes are membrane bound or associated and it is very likely that at least some of these phospholipases are regulatory enzymes. There has been increasing interest recently in the isolation and characterization of these intracellular phospholipase activities. For example, much attention has been focused on the phosphatidyl-inositol (PI) specific phospholipase C which has been implicated as a participant in the "PI response" (Marx, 1985). There has also been much interest in the role of phospholipases in the regulation of eicosanoid production (Smith and Borgeat, 1985).

A group of cellular phospholipases which may share some of the properties of both the extracellular and intracellular lipases are the lysosomal phospholipases. These enzymes operate in an acid environment, can be isolated in soluble form, and are believed to function in the degradation of phospholipids which enter the cell via endocytic processes. However, it is not known if these enzymes are membrane-associated within the cell or how they are regulated. In general, little is known of how the intracellular degradation of phospholipids is controlled. These studies have not advanced as rapidly as those dealing with the catabolism of water soluble compounds. Phospholipids are difficult enzyme substrates to use and phospholipase kinetics are very complex. As noted, many phospholipases are membrane associated, making them difficult to assay, purify, and characterize. Still in recent years considerable progress has been made in

the isolation and characterization of lysosomal phospholipases, particularly those of rat liver.

1.4.3.1 Phospholipase A

The first evidence of phospholipase A activity in liver lysosomes came from the laboratory of Mellors. It was demonstrated that coincubation of lysosomal fractions and mitochondria resulted in uncoupling of oxidative phosphorylation, due to the production of free fatty acids by a lysosomal phospholipase (Mellors *et al.*, 1967; Mellors and Tappel, 1967). At the same time, a rat liver lysosomal phospholipase A activity was described by Stoffel and Greten (1967). Through the use of radioactive phospholipid substrates, labelled specifically in either the C1 or C2 fatty acyl chain, it was possible to demonstrate that phospholipase activities specific for both positions were present in liver lysosomes (Waite *et al.*, 1967; Stoffel and Trabert, 1969; Fowler and de Duve, 1969; Nachbaur *et al.*, 1972).

The separation of the lysosomal phospholipase A_1, and phospholipase A_2 activities from each other, and the purification and characterization of the proteins responsible has been a challenging task. Waite and coworkers reported the first partial separation of the two phospholipase activities in 1971 (Franson *et al.*, 1971). More recently this group has reported purifications of rat liver lysosomal phospholipases A_1 and A_2 (Waite *et al.*, 1976; Robinson and Waite, 1983). Lysosomal phospholipase A has also been purified by Hostetler *et al.* (1982), who reported five isoenzymes with phospholipase A_1 and lysophospholipase activities.

There are considerable and as yet unresolved differences in the properties of the phospholipase A activities isolated in the laboratories of Waite and Hostetler. The enzyme described by Waite and coworkers (Waite *et al.*, 1976; Waite *et al.*, 1981; Robinson and Waite, 1983) has been purified 1,440-fold from the soluble fraction of rat liver lysosomes. It has optinal activity at pH 4.0, and a molecular weight of 56,000 daltons as determined by denaturing polyacrylamide gel electrophoresis. The enzyme binds ConA and is therefore believed to be a glycoprotein. *In vitro*, phosphatidyl ethanolamine (PE) is the preferred substrate of the enzyme; PC is hydrolyzed at 20% of the rate of PE and acidic

phospholipids (PS, PI, and PG) are all poor substrates.

The five isoenzymes of acid phospholipase A purified by Hostetler *et al.* (1982) from the soluble fraction of a rat liver composite lysosomal fraction have molecular weights ranging from 15,000 to 90,000 daltons, as determined by gel filtration. The two major forms have molecular weights of 34,000 and 44,000 daltons and have been purified 17,500-fold and 1,910-fold, respectively. All the enzymes are glycoproteins and bind to ConA-Sepharose. Neither of the major isoenzymes was inhibited by Ca^{2+} or Na^{+}, in contrast to the enzyme reported by Waite and coworkers. The enzymes showed no phospholipase A_2 activity, but demonstrated lysophospholipase A activity. In the presence of Triton X-100, the major isoenzymes will hydrolyze acidic as well as neutral phospholipids, again in contrast to the data of Waite and coworkers. Phosphatidylglycerol and cardiolipin are the preferred substrates, followed by PC, PI, PS, and PE.

1.4.3.2 Phospholipase C

Two different rat liver lysosomal phospholipase C activities have been reported. Dawson and coworkers (Irvine *et al.*, 1977a, 1977b, 1978) described a lysosomal enzyme that cleaves inositol monophosphate from PI. When assayed with pure lipid substrates, this activity showed negligible activity with phospholipids other than PI. In addition to this PI-specific phospholipase C, liver lysosomal phospholipase C showing broad substrate specificity has been described by Matsuzawa and Hostetler (1980). When assayed with pure lipid substrates, this enzyme was most active against PI, but also showed substantial activity with PC or PG, and significant activity against PS and PE.

1.4.3.3 Sphingomyelinase

Sphingomyelin can be degraded to ceramide and phosphorylcholine by a phospholipase C - like enzyme called sphingomyelinase. Sphingomyelinase occurs in various tissues, most often as a lysosomal component (Yamaguchi and Suzuki, 1977). Neutral forms have also been reported in microsomes (Gatt, 1976; Rao and Spence, 1976) and plasma

membrane (Hostetler and Yazaki, 1979). The solubilization of sphingomyelinase requires detergents, implying that it is in some way membrane associated. *In vitro*, the reaction kinetics of sphingomyelinase are influenced by the presence of detergents, other phospholipids, and cholesterol (Brady, 1983).

There are several clinical disorders, collectively referred to as Niemann-Pick's disease, which are characterized by accumulation of sphingomyelin (Kanfer and Hakomori, 1983). In two forms of the disease, type A and type B, this accumulation is associated with reduced levels of lysosomal sphingomyelinase. In type C disease, however, sphingomyelinase levels appear to be normal while sphingomyelin accumulates to 3-5 times its normal levels in liver and spleen. It is difficult to reconcile this accumulation with the apparent abundance of enzyme activity, but this situation is not unique. There are many circumstances, particularly with cells grown in culture, where abnormal lysosomes appear, yet there are little or no signs of intrinsic defects in the complement of acid hydrolases (Phillipart, 1975). It may be possible that there are some lysosomal storage disorders in which the hydrolases may be normally active *in vitro* but not *in vivo*, due to some lysosomal dysfunction involving for example its membrane, matrix, pH, or cofactors.

1.5 Intracellular Lipid Trafficking and Metabolism

1.5.1 Introduction

Membrane lipid composition is without question regulated. Tissues in different species having identical function contain remarkably similar lipids. Conversely, tissues within the same organism which have different functions are distinctly different from each other in their lipid composition. At the subcellular level, it is now well documented that the membranes of various organelles differ widely from one type of functional membrane to another (Thompson, 1980; Dandrifosse, 1985). In particular, the sterol content varies dramatically from one intracellular membrane to another. The plasma membrane is characteristically sterol rich, containing close to

equimolar amounts of cholesterol and phospholipid (Thompson, 1980). Other intracellular organelles, such as mitochondria and endoplasmic reticulum, are typically isolated as membranes containing much less sterol (Hashimoto *et al.*, 1983). How this separation is maintained, in view of the known mobility of cholesterol, remains unknown.

Phospholipids also demonstrate a somewhat heterogeneous distribution throughout the different cellular membranes. Sphingomyelin is enriched in the plasma membrane and is virtually absent from mitochondria, while phosphatidylcholine is widely distributed, but is enriched slightly in the endoplasmic reticulum. Cardiolipin appears to be exclusively located in mitochondria (Thompson, 1980). How these differences in lipid composition are maintained is unknown.

1.5.2 Intracellular Lipid Movement

The enzymes of lipid synthesis are found largely, but not exclusively, in the microsomal subcellular fraction. Cholesterol and phosphatidylcholine are synthesized *de novo* in the endoplasmic reticulum and there must therefore be mechanisms for transport and distribution of new lipids to remote and functionally distinct membranes elsewhere in the cell. Remarkably little is known about the specifity, mechanisms, or regulation of intracellular lipid movements.

Only recently have investigators begun to study the intracellular movements of cholesterol. DeGrella and Simoni (1982) demonstrated that in Chinese hamster ovary cells newly synthesized cholesterol is translocated from the endoplasmic reticulum to the plasma membrane quite rapidly, with a half time of about 10 minutes. In amoeba, sterol transport to the cell surface occurs within 30 minutes (Mills *et al.*, 1984). In contrast, however, the movement of cholesterol from the plasma membrane to the cell interior seems to be a much more slower or limited process. Cholesterol introduced into the plasma membrane of human fibroblasts via exchange does not readily equilibrate with endogenously synthesized cholesterol (Poznansky and Czekanski, 1982) nor does it become rapidly available for microsomal esterification (Poznansky and Czekanski, 1982; Shireman and Remsen, 1982). In rat smooth muscle cells

there is a similar situation; only 2-3% of cholesterol introduced via exchange from lipid vesicles is esterified within 24 hours (Slotte and Lundberg, 1983*a*). Kaplan *et al.* (1984) have also reported that newly synthesized cholesterol, once transported to the plasma membrane, remains there and does not return to the cell interior.

The mechanisms for intracellular transport of phospholipids and cholesterol from their sites of synthesis to the different organelle membranes throughout the cell are unknown. Various potential mechanisms can be envisaged, and there is at least circumstantial evidence for several (reviewed by Voelker, 1985; Green, 1983). Lipids could simply move by aqueous diffusion mechanisms. Certainly free fatty acids, phosphatidic acid, and probably cholesterol have sufficient aqueous monomer solubility to transfer through the cytosol in this manner. However, the existence of several cytosolic lipid-binding proteins have been demonstrated (discussed below) and it is likely that these proteins play important roles in intracellular lipid transport.

There is also considerable morphological evidence for other potential mechanisms of lipid transport via vesicular trafficking or membrane-membrane fusion processes, but as yet it has been difficult to design experiments which can distinguish between this kind of transport and lipid movements which occur by diffusion or exchange proteins.

1.5.2.1 Sterol carrier protein

SCP is a highly abundant, ubiquitous protein which has multiple roles in the regulation of lipid metabolism (Dempsey, M.E., 1984). The same protein has been variously described by different groups as liver fatty acid binding protein (FABP) (Ockner *et al*, 1982; Gordon *et al.*, 1983), amino azo dye-binding protein A (Ketter *et al.*, 1976), and band C (Billheimer and Gaylor, 1980). This multiplicity of names is testament to the fact it is a usual protein which serves as a cofactor for a number of membrane bound enzymes catalyzing specific steps in lipid metabolism and which also seems to be involved in the intracellular movement of cholesterol and fatty acids. SCP is widely distributed in the cytosolic fraction of mammalian tissues and is also present in various cultured cells. In

cultured human skin fibroblasts, SCP levels respond positively to the presence of lipid in the culture medium (Dempsey *et al.*, 1982; Alley *et al.*, 1982), perhaps implying a role for this protein in the delivery or utilization of exogenous lipids by cells grown in culture.

SCP is absolutely required for the activation of membrane-bound enzymes needed for the biosynthesis of cholesterol from lanosterol (Dempsey, 1984). It also participates in metabolism of cholesterol to bile acids (Grabowski *et al.*, 1976) as well as in the synthesis of steroid hormones (Conneely *et al.*, 1984).

SCP also has a high affinity for long chain fatty acids and is believed to function as an intracellular fatty acid transporter (analogous to albumin which is the fatty acid carrier in the plasma). SCP will also activate ACAT (Dempsey, 1984). Other work has shown that SCP will stimulate utilization of fatty acids for phospholipid and triacylglcerol synthesis (Ockner *et al.*, 1982). SCP undergoes a rapid turnover *in vivo*, a characteristic expected for a regulatory protein. Another related protein is a fatty acid binding protein similar to the nonspecific lipid transfer protein (Bloj and Zilversmit, 1977; Poorthuis *et al.*, 1981).

1.5.2.2 Phospholipid exchange proteins

Proteins that catalyze the exchange or transfer of phospholipids between various combinations of synthetic lipid membranes, lipoproteins, cellular membranes and organelles have been isolated from the cytosolic fraction of many mammalian tissues (Wirtz, 1974; Zilversmit and Hughes, 1976). Exchange proteins which have particular specificities for certain headgroups (for example PC or phosphatidylinositol, PI) have been described, while other so called "nonspecific" transfer proteins will transfer cholesterol as well as various phospholipids (Bloj and Zilversmit, 1977). The role played by these proteins *in vivo* is unknown; it is possible that they may function in membrane biogenesis or that they may be involved in tailoring the lipid composition of different organelle membranes.

1.6 Objectives

The overall objective of this study was to investigate two different types of lipid movements between serum components and cultured human skin fibroblasts:

1. non-receptor mediated movements of free cholesterol,
2. receptor mediated uptake of LDL-associated PC.

In both cases, the effects of these movements on aspects of intracellular lipid metabolism were determined, and the intracellular movements of these lipids were investigated.

In the first series of experiments the role of non-receptor mediated movements of cholesterol in the regulation of cholesterol homeostasis in cultured fibroblasts was investigated. These experiments were carried out in the absence of serum in order to avoid complicating additional fluxes of cholesterol via the LDL receptor pathway or via exchange to or from other serum components. Unilamellar lipid vesicles of various cholesterol/PC compositions were used to alter, via passive exchange, the cellular cholesterol content. The effects of these alterations on cellular cholesterol synthesis, cholesterol esterification, and LDL binding were determined.

In the second series of experiments the uptake and intracellular fate of LDL-associated PC was investigated. A procedure was developed for the incorporation of radiolabelled PC into a biologically active reconstituted LDL particle. This reconstituted LDL was taken up by the LDL receptor pathway of cultured fibroblasts, and the catabolic fate of its various components was determined. As well, the effect of delivery of PC to cells via the LDL pathway on cellular PC biosynthesis was investigated.

2. MATERIALS AND METHODS

2.1 Materials

[1,2-^{3}H]cholesterol, [cholesteryl-1,2,6,7-^{3}H]cholesteryl linoleate (46 Ci/mmol), [oleate-1-^{14}C]cholesteryl oleate, [1-^{14}C]oleic acid, [cholesteryl-1,2-^{3}H]cholesteryl hexadecyl ether (46.8 Ci/mmol), [1-^{14}C]acetic acid, [2-palmitoyl-9,10-^{3}H]and [choline-methyl-^{3}H]dipalmitoyl phosphatidylcholine (48 Ci/mmol), and [dioleoyl-1-^{14}C] (114 mCi/mmol) dioleoyl phosphatidylcholine were obtained from New England Nuclear (Lachine, Quebec). [dioleoyl-1-^{3}H]dioleoyl phosphatidylcholine (9.6 Ci/mol) was prepared by the method of Patel *et al.* (1979). Na [^{125}I]iodide (carrier free) was obtained from the Edmonton Radiopharmaceutical Center (Edmonton, Alberta). Aqueous counting scintillant was purchased from Amersham Canada (Oakville, Ontario). Phosphatidylcholine (Type XI-E, from fresh egg yolk), synthetic dioleoyl phosphatidylcholine, cholesteryl linoleate, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Cholesterol was recrystallized from ethanol before use. Purified potato starch powder was purchased from Fisher Scientific Co. Sepharose 4B was obtained from Pharmacia. Chloroform, methanol, hexane, and heptane were purchased from Fisher Scientific Co. and were redistilled prior to use. Fetal calf serum was purchased from Flow Laboratories (Toronto, Ontario). Tissue culture media and LUX plasticware were obtained from Flow Laboratories or Gibco Canada (Burlington, Ontario). Other chemicals were from Sigma Chemical Co. or Fisher Scientific Co. Sil G plastic backed thin layer chromatography plates were purchased from Brinkmann Canada (Rexdale, Ontario). Dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane) was obtained from BDH Chemicals, Canada (Edmonton, AB). Gas liquid chromatography supplies were from Supleco (Oakville, Ontario).

2.2 Human Skin Fibroblasts

Normal human skin fibroblasts (Detroit 551) were obtained from the American Type Culture Collection (Rockville, MD) and FH fibroblasts (GM 1915) from a patient with homozygous familial hypercholesterolemia were obtained from the Human Mutant Cell Repository (Camden, NJ). The cells were used between the fourth and twelfth passages, and were cultured in Eagle's minimum essential medium (MEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 24 mM-$NaH\text{-}CO_3$ and 10% (v/v) fetal bovine serum. The cells were plated at a density of 5 x 10^5 cells/25 cm^2 flask on day 0, and fresh medium was added on day 3. On day 6 the growth medium was replaced with medium which contained 10% (v/v) human LPDS and the confluent cell cultures were used 24 hours later on day 7. For some experiments the cells were grown in medium containing 10% fetal bovine serum for the final 24 hours.

2.3 Lipoproteins

Human LDL (density 1.091-1.063 g/ml) and human LPDS (density > 1.215 g/ml) were prepared by differential density ultracentrifugation of human plasma obtained from healthy volunteers (Hatch and Lees, 1968). Venous blood (450 ml) was collected in a plastic centrifuge bottle containing disodium EDTA as an anticoagulant. The plasma was isolated by low speed centrifugation (4,000 x g for 30 minutes at 4°C) and was used immediately for lipoprotein isolation. The plasma was adjusted to a final density of 1.019 g/ml by the addition of solid potassium bromide and then centrifuged in the 70 Ti rotor of a Beckman ultracentrifuge at 50,000 rpm for 20 hours at 4°C. The upper milky layer containing VLDL was discarded and the remaining sample was adjusted to a density of 1.063 g/ml with potassium bromide and then centrifuged at 50,000 rpm for 28 hours at 4°C. The LDL was collected from the top layer of the tubes and the remainder of the sample was adjusted to a density of 1.215 g/ml. Following centrifugation at 55,000 rpm for 48 hours, the LPDS fraction was isolated by puncturing the bottom of the tubes. The isolated fractions were dialyzed against 0.15 M-NaCl/0.3 mM-EDTA

(pH 7.4) and sterilized by filtration prior to use. Protein concentrations were determined by the method of Lowry *et al.* (1951). ^{125}I-LDL was prepared by the iodine monochloride method of Macfarlane (1958) as modified by Bilheimer *et al.* (1972) and described by Goldstein *et al.* (1983).

2.4 Lipid Vesicles

To prepare unilamellar vesicles, various amounts of cholesterol were added to 10 mg of egg phosphatidylcholine to produce vesicles with C/P ratios of 0, 0.6, and 2. The lipids were co-lyophilized from benzene, dispersed in 10 ml of Eagle's minimum essential medium and sonicated at 4°C under N_2 with a Branson W185 probe sonicator as described previously (Poznansky and Czekanski, 1982). For some experiments, tracer amounts of [^{14}C]cholesteryl oleate or [^{3}H]dioleoyl phosphatidylcholine were added to the lipids prior to lyophilization. Greater than 90% of the vesicle lipid was in the unilamellar form, as determined by its elution profile following gel filtration chromatography on Sepharose 4B. Gas liquid chromatography of vesicle lipids revealed no breakdown or oxidation products.

2.5 Cholesterol and Phosphate Determinations

Cell lipids were extracted according to the procedure of Bligh and Dyer (1959), and lipid phosphorus was determined as described by Chalvardjian and Rudnicki (1970). For calculations of phospholipid mass, it was assumed that phosphorous constitutes 4% of the total phospholipid weight. Cell free cholesterol was quantified on a Hewlett Packard 5730A gas chromatograph with 3% OV-17 columns operated isothermally at 250°C and with 5α-cholestane as an internal standard. Lipoprotein cholesterol was determined chemically by the method of Rudel and Morris (1973).

2.6 Incubation of Cells with Lipid Vesicles

On day 7 following a 24 hour incubation in MEM containing 10% LPDS, cell monolayers were washed with Dulbecco's phosphate buffered saline (PBS) and the medium was replaced with 2 ml of minimum essential medium which contained the lipid vesicles. The final concentration of the vesicle phospholipid was 0.5 mg/ml, which was a 10-15 fold excess over cell phospholipid. The cells were incubated in the presence of the vesicles for 6 or 24 hours at 37°C before determinations of LDL binding, cholesterol synthesis and cholesterol esterification were made. For some control experiments, various concentrations of vesicles containing tracer amounts of [^{3}H] cholesterol, [^{14}C] cholesteryl oleate, or [^{3}H] phosphatidylcholine were incubated with cells in the presence of different amounts of bovine serum albumin (BSA).

2.7 Acetate Incorporation into Cholesterol

After the experimental incubation of 6 or 24 hours, 10 μCi of [^{14}C]acetate (New England Nuclear) was added to each flask and the incubation was continued at 37°C for 2 hours. The cells were washed five times with phosphate-buffered saline and were harvested from the flasks by scraping with a rubber policeman or with 0.25% trypsin. The lipids were extracted and the radiolabelled cholesterol was isolated by TLC on Sil G plastic backed plates (Brinkmann Canada, Rexdale, Ontario, Canada), which were developed in hexane/diethyl ether/acetic acid (85:20:2, by vol.). The incorporation of labelled acetate into cholesterol was determined by liquid-scintillation counting of the cholesterol spot and was corrected for total cell protein. The neutral lipids identified on the plates, with their corresponding R_f values, were polar lipids, 0; monoglycerides, 0.10; diglycerides, 0.15; cholesterol, 0.28; free fatty acids, 0.35; triglycerides, 0.65; and cholesteryl esters, 0.9.

2.8 Oleate Incorporation into Cholesteryl Esters

Cell monolayers were incubated with 10 μCi of [^{3}H]oleate for 4 hours at 37°C. The cells were harvested as above, their lipids were extracted and were chromatographed on Sil G plates. The incorporation of labelled oleate into cholesteryl esters was determined by liquid-scintillation counting of the cholesteryl ester spot, and was corrected for total cell protein.

2.9 Binding of LDL

The binding of ^{125}I-LDL was determined as described by Brown and Goldstein (1975). Following the experimental incubations, the medium was removed and the monolayers were washed with cold Dulbecco's phosphate-buffered saline. Then 2 ml of cold MEM containing 10% LPDS and 5 μg of ^{125}I-LDL/ml were added to each flask and the flasks were incubated with rotary shaking at 4°C for 2 hours. The medium was then removed and the monolayers were washed extensively. After addition of 1 ml of 0.1 M-NaOH to the cells, samples were removed for determination of cell protein and of ^{125}I-LDL radioactivity (in a Beckman Gamma 4000 counter). All ^{125}I-LDL binding data were corrected for non-specific binding which was determined from the binding assay done in the presence of 250 μg of unlabelled LDL/ml.

2.10 Preparation of Reconstituted LDL Particles

Reconstituted LDL particles containing various radiolabelled lipids were prepared from either native LDL or ^{125}I-LDL by a modification of the procedure of Krieger *et al.* (1978). 50 mg of LDL protein in 15 ml of 0.15 M NaCl and 0.2 mM Na_2 EDTA, pH 7.4, were dialyzed for 60 hours at 4°C against 6 changes of 4 liters of 0.3 mM Na_2 EDTA, pH 7.0. Aliquots of dialyzed LDL containing 1.9 mg of protein were added to 13 x 100 mm glass test tubes which had been previously treated with 2% dimethyldichlorosilane and which each contained 25 mg purified potato starch. The LDL-potato starch suspension was quick-frozen in liquid N_2, lyophilized for 6 hours, and stored overnight at 4°C under vacuum over phosphorous pentoxide.

Each tube was extracted for 1 hour with 5 ml heptane at -10°C. The potato starch-LDL protein complex was separated from the heptane supernatant by centrifugation at maximum speed for 15 min at 4°C in an International Clinical Centrifuge (Fisher Scientific). The neutral lipid-containing heptane supernatant was discarded and the extraction procedure was repeated two more times. Following the removal of the last heptane supernatant, 200 μl of heptane containing 6 mg of cholesteryl linoleate and tracer amounts of either radiolabelled CE or cholesteryl ether, and/or radiolabelled PC, was added to each tube. For some experiments various amounts of non-radioactive egg PC or DOPC were included in the 200 μl of heptane. After incubation of the heptane/starch/LDL suspension for 1.5 hours at -10°C, the heptane was evaporated on ice under N_2 until the pellet was powder dry. The reconstituted LDL particle was solubilized by the addition to each tube of 1 ml of 10 mM Tricine pH 8.4 followed by a 41 hours incubation at 4°C. Each suspension was transferred to a 1.5 ml plastic microfuge tube and centrifuged in a Beckman microfuge (12,000 rpm) for 20 min at 4°C. The soluble reconstituted LDL in the supernatant was sterilized by passage through a 0.45 μm filter; this also removed any residual particulate material. Radiolabelled free cholesterol was incorporated into the reconstituted LDL by the method of Lundberg *et al.* (1982).

.2.11 Incubation of Cells with Reconstituted LDL

Typically, cells were grown in minimum essential medium containing 10% LPDS for 24 hours before use. For some experiments, this preincubation was not carried out. Cell monolayers were washed with Dulbecco's PBS and the medium was replaced with medium containing the reconstituted LDL. The concentration of reconstituted LDL was typically 20 μg/ml, which is approximately half-maximal for LDL binding at 37°C (Goldstein *et al.*, 1983). Following incubation at 37°C for various times up to 8 hours, the medium was removed and the cell monolayers washed five times with either Dulbecco's PBS or Hank's balanced salt solution. The cells were harvested by scraping with a rubber policeman. Normally the cellular lipids were extracted according to the method of Bligh and Dyer (1959) and subjected to neutral lipid or

polar lipid TLC. The neutral TLC solvent system is described in section 2.7. For TLC of polar lipids, the lipid extract was applied to Sil G plastic backed plates (Brinkmann) which were developed in chloroform/methanol/acetic acid/water (50:25:7:3, by volume). The R*f* values for the various polar lipids in this solvent system were as follows: lysophosphatidylcholine, 0.10; sphingomyelin, 0.17; phosphatidylcholine, 0.30; phosphatidylserine, 0.61; phosphatidylinositol, 0.51; phosphatidylethanolamine, 0.83. Neutral lipids ran with the solvent front. Following chromatography, the plates were cut into strips and counted in 15 ml aqueous counting scintillant in a Beckman liquid scintillation counter. For some experiments, the lipids were eluted from the TLC plates by repeated extraction of the silica gel with chloroform/methanol (2:1).

2.12 Hydrolysis of ^{125}I-LDL by Cells

The measurement of the proteolytic degradation of ^{125}I-LDL and reconstituted ^{125}I-LDL by fibroblasts was carried out essentially as described by Goldstein *et al.* (1983). Following incubation of the cells with ^{125}I-LDL for various times, the medium was removed and precipitated with a final concentration of 10% trichloroacetic acid for 1 hour at 4°C. This precipitated intact ^{125}I-LDL, which was removed by centrifugation. The acid-soluble supernatant was treated with 30% hydrogen peroxide (40 μl per ml of supernatant). This converts any ^{125}I-iodide ions to ^{125}I-iodine, which was extracted by the addition of 2 vol. chloroform followed by mixing and separation of the aqueous and chloroform layers. An aliquot of the aqueous layer was counted for ^{125}I radioactivity in a Beckman Gamma 4000 counter. It has been shown previously (Goldstein and Brown, 1974) that this aqueous radioactivity consists of ^{125}I-monoiodotyrosine formed by the hydrolysis of LDL in lysosomes.

2.13 TLC of Osmium Tetroxide Treated Lipid Extracts

In order to separate DPPC from other PC species by TLC, lipid extracts were first treated for 15 minutes with osmium tetroxide in carbon tetrachloride (0.6 mg/ml) (Mason *et al.*, 1976). Approximately a ten-fold excess of osmium tetroxide over lipid (by weight) was used. The samples were dried, resuspended in chloroform and run in the regular polar TLC solvent system. Osmium treated samples showed a dark deposit on the TLC plate which migrated slightly from the origin but it did not affect the resolution of the PC spot, nor did it appear to reduce liquid scintillation counting efficiency.

2.14 Statistical Analysis of the Data

Data were analyzed by a one-way analysis of variance and statistical significance was determined by the Duncan-Bonnor test (Duncan, 1955). Statistical significance was accepted if the P value was < 0.05.

3. RESULTS

3.1 Preparation of LDL, LPDS, and ^{125}I-LDL

LDL and LPDS were prepared by sequential ultracentrifugation of fresh human plasma. Typically about 30 ml of LDL at a protein concentration of approximately 4 mg/ml were obtained from 450 ml of blood. The LDL had equal amounts of total cholesterol and protein, as indicated in Table 1. This is a typical result for isolated native LDL (Krieger *et al.*, 1978). The LPDS isolated by this method contained very little cholesterol, averaging 0.22 μg per mg of protein. When the LPDS was diluted to the original plasma volume, this equalled 15 μg of cholesterol per ml (Table 1). This represents less than 1% of the cholesterol normally present in serum.

The lipid composition of the isolated LDL was determined. LDL lipids were extracted and separated by neutral and polar thin layer chromatography and the concentrations of the LDL lipid components were determined (Table 2). The LDL contained a total of 1.04 mg cholesterol per mg of protein, three quarters of which was in the cholesteryl ester form, with 25% as free cholesterol. The content of LDL triacylglycerol was not determined, but from the amount visualized on the TLC plate by staining with I_2 vapor it appeared that it represented much less than 5% of the stained neutral lipid material. These results are consistent with other studies showing that the neutral lipids of LDL consist primarily of cholesteryl esters, with some free cholesterol and very few triacylglycerol molecules (Lee, 1976).

LDL was iodinated by the iodine monochloride method (Bilheimer *et al.*, 1972). In general, the final specific activity of the ^{125}I-LDL was from 250 to 400 cpm/ng protein. Less than 2% of the ^{125}I-LDL remained soluble following protein precipitation with 10% trichloroacetic acid, and less than 5% of the radioactivity could be removed by a lipid extraction procedure (Table 3).

The binding of ^{125}I-LDL to normal human skin fibroblasts was investigated. In the first experiment, the concentration dependence of ^{125}I-LDL cell surface binding at 4°C was

Table 1. Protein and cholesterol content of human LDL and LPDS. LDL and LPDS were prepared by sequential ultracentrifugation of approximately 200 ml of human plasma. Data are the means ± 1 S.D. for fifteen different preparations.

	Protein (mg/ml)	Cholesterol (mg/ml)	Cholesterol/Protein (mg/mg)
LDL	4.12 ± 1.08	4.04 ± 1.34	0.98 ± 0.13
LPDS	67.6 ± 16.3	0.015 ± 0.0052	0.00022 ± 0.00007

Table 2. Lipid composition of LDL. LDL lipids were extracted and chromatographed as described in Materials and Methods. The lipids were eluted from the tlc plates and their concentrations determined by chemical assay as described under Materials and Methods. Each value represents the mean of duplicate determinations.

Neutral Thin Layer Chromatography

Component	Concentration (mg/mg protein)
Free cholesterol	0.28
Cholesteryl esters	0.74
Triglycerides	not determined

Polar Thin Layer Chromatography

Component	Concentration (mg/mg protein)
PC	0.57
SM	0.18
PE	< 0.05
PS	< 0.05
lyso PC	< 0.05

Table 3. Characterization of ^{125}I-LDL. 2.5 µg of LDL protein was either precipitated with 10% trichloroacetic acid or extracted with methanol/chloroform as described under Materials and Methods.

TCA Precipitation	
total cpm added	688143
total cpm remaining in supernatant	13218 (1.90%)
Lipid Extraction	
total cpm added	688143
total cpm extracted	33530 (4.87%)

determined. The cells were preincubated for 24 hours in LPDS to induce maximal LDL receptor activity. At 4°C receptor mediated endocytosis of LDL does not occur (Goldstein *et al.*, 1983), and the amount of radioactive LDL associated with the cells is a good measure of the cell surface binding. As shown in Figure 5, binding of ^{125}I-LDL at 4°C reaches a maximum of 115 ng/mg cell protein at an LDL concentration of approximately 25 μg/ml. Half maximal binding occurs at 2.5 μg/ml. Non-specific binding determined in the presence of 250 μg/ml LDL was always less than 10% of the total binding.

The binding of ^{125}I-LDL to fibroblast monolayers at 4°C as a function of time was also determined (Figure 6). Binding of ^{125}I-LDL to normal cells preincubated for 24 hours in LPDS plateaued after about 2 hours at approximately 120 ng/mg of cell protein. Binding to normal fibroblasts which had been grown exclusively in the presence of fetal calf serum also reached a maximum after 2 hours incubation but the total binding was only 15 ng/mg cell protein. This down regulation of LDL receptor activity in cells grown in the presence of whole serum is a well known phenomenon (Goldstein and Brown, 1977). Fibroblasts from a familial hypercholesterolemia homozygote (FH), which lack functional LDL receptors, showed only background binding of ^{125}I-LDL. From these results it can be concluded that the ^{125}I-LDL is recognized by the fibroblast LDL receptor described by Goldstein, Brown and coworkers.

3.2 Nonreceptor Mediated Cholesterol Movements

3.2.1 Cholesterol exchange between vesicles and fibroblasts

Since lipid vesicles were going to be used to alter fibroblast cholesterol content, suitable conditions for cholesterol exchange between vesicles and cells were determined. Lipid vesicles with C/P = 0.6 were prepared containing tracer amounts of [^{3}H]cholesterol and [^{14}C]cholesteryl oleate, which served as a non-exchangeable vesicle marker. Fractionation of the vesicles by gel filtration on a Sepharose 4B column demonstrated that more than 90% of the lipids and radioactivity co-eluted as unilamellar vesicles in the included volume. Gas liquid

Figure 5. Concentration dependence of cell surface binding of ^{125}I-LDL to normal fibroblasts. The cells were incubated in medium containing 10% LPDS for 24 hours before the binding assay was performed. Fibroblasts were incubated with various concentrations of ^{125}I-LDL for 2 hours at 4°C. The binding was determined as described under Materials and Methods. Specific binding (Specific) was calculated by subtraction of nonspecific binding, determined in the presence of 250 μg/ml non-radioactive LDL, (Nonspecific) from the mean of triplicate (Total) or duplicate (Nonspecific) samples.

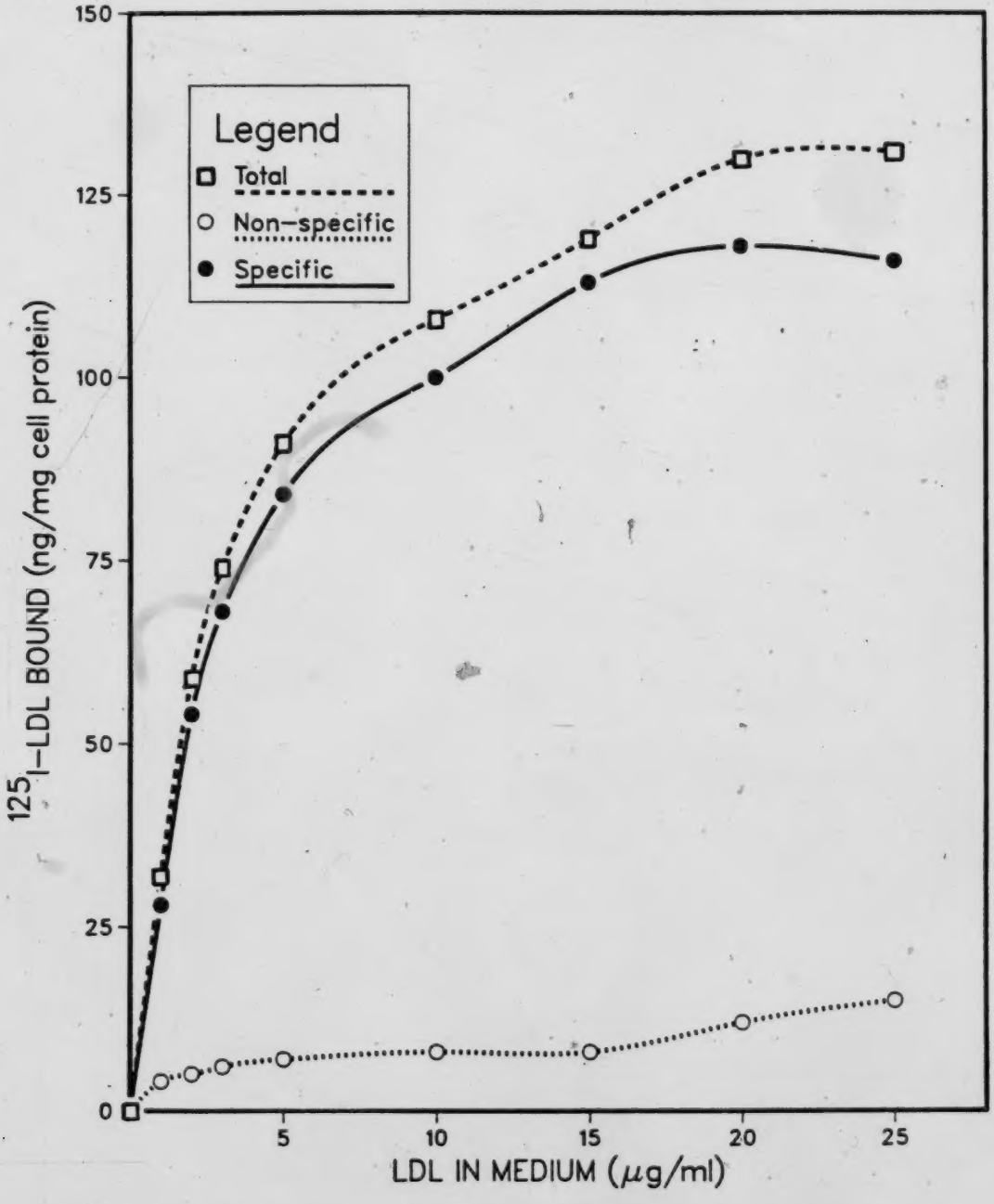
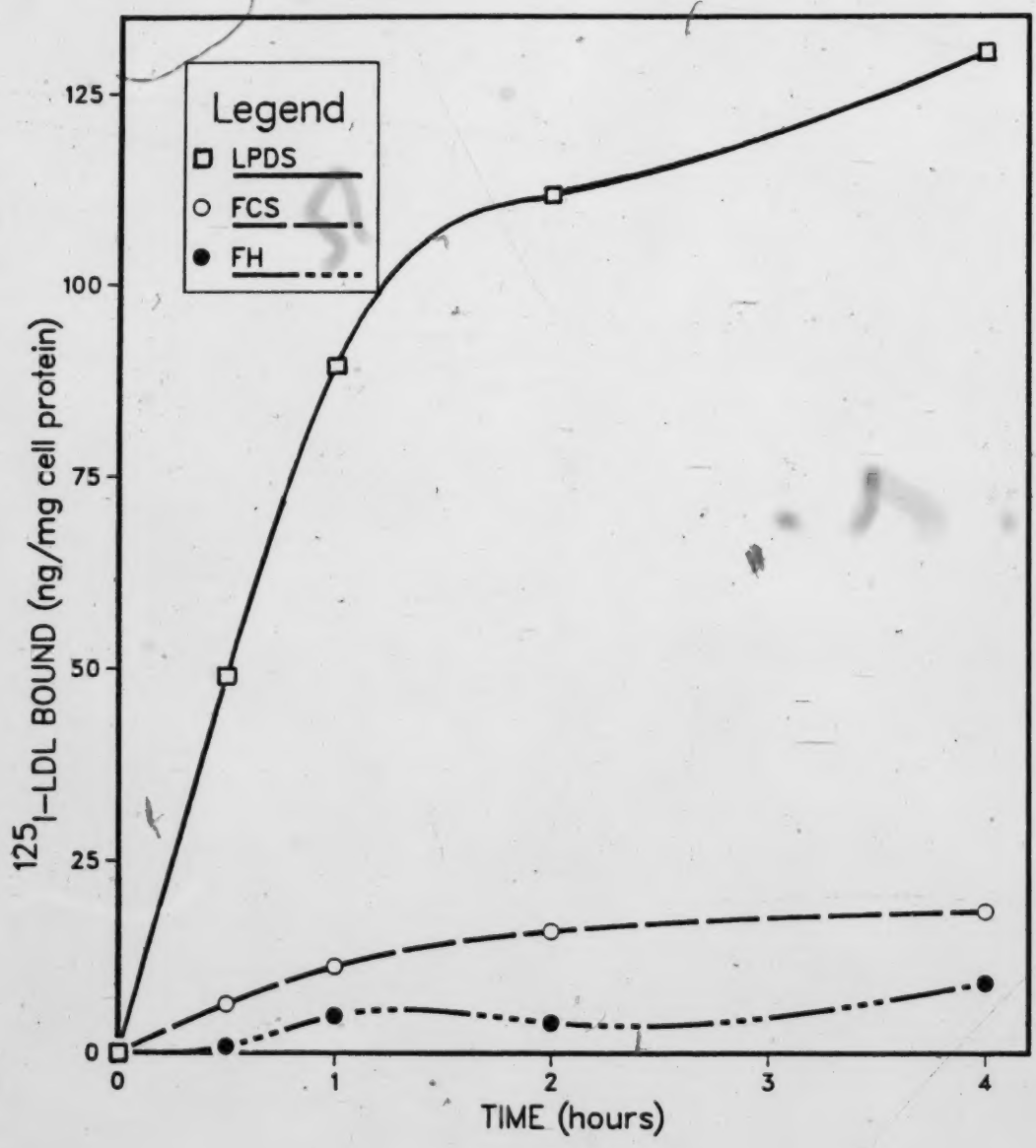


Figure 6. Time dependence of binding of ^{125}I-LDL to fibroblasts at 4°C. Normal human skin fibroblasts which had been preincubated for 24 hours in 10% LPDS (LPDS) or 10% FCS (FCS), and fibroblasts from a familial hypercholesterolemia homozygote (FH) were incubated with 5 μg/ml ^{125}I-LDL for various times at 4°C. The binding assay was performed as described under Materials and Methods. Each value represents the mean of triplicate samples which have been corrected for nonspecific binding.



chromatographic traces of extracted vesicle lipids showed no evidence of oxidized lipids. Various concentrations of the vesicles were incubated with normal fibroblasts for 2 hours at 37°C, and the exchange of radioactivity into the cells was determined (Figure 7). There was essentially no uptake of [^{14}C]cholesteryl ester by the cells for all vesicle concentrations (data not shown). The [^{3}H]cholesterol readily exchanged from the vesicles to the fibroblasts and a cholesterol concentration of 50 μg/ml appeared to be optimal for this process. Since the fibroblast monolayers contained approximately 8 μg of cholesterol per 25 cm^2 flask, this vesicle cholesterol concentration represents a 12.5 fold excess over cell cholesterol.

Since it has been reported that serum albumin may facilitate cholesterol exchange with mammalian cells (Bartholow and Geyer, 1981, 1982), the effect of different concentrations of albumin on the exchange of [^{3}H]cholesterol between lipid vesicles and fibroblasts was investigated (Figure 8). Although some stimulation of exchange was evident in the presence of albumin, it was not nearly as great as reported by Bartholow and Geyer (1982) and it was decided to carry out subsequent experiments in the absence of albumin (or any other serum proteins).

3.2.2 Effect of cholesterol exchange on fibroblast cholesterol content

Fibroblast monolayers were incubated for 24 hours in MEM containing 10% lipoprotein deficient serum (LPDS). The cells were then exposed for 6 to 24 hours to medium containing LDL, or lipid vesicles with cholesterol/phospholipid (C/P) molar ratios of 0, 0.6, or 2. Cell morphology remained normal during all incubations and viability, as assessed by exclusion of 0.5% nigrosin, remained $>$ 90% for up to 24 hours (Table 4). Cell protein averaged 250 μg per flask and this did not vary significantly with different incubation conditions. To ensure that cholesterol movements from the vesicles into the cells occurred via exchange at the plasma membrane rather than via fluid phase pinocytosis or vesicle-cell fusion, control incubations were carried out with vesicles containing tracer amounts of ^{14}C-cholesteryl oleate, a non-exchangeable marker. Even with prolonged incubations of up to 24 hours, less than 0.1%

Figure 7. Exchange of [^{3}H] cholesterol between vesicles and fibroblasts. Egg PC/cholesterol vesicles (C/P = 0.6) containing tracer amounts of [^{3}H] cholesterol and [^{14}C] cholesteryl oleate were incubated in 2 ml medium with normal fibroblasts for 2 hours at 37°C. Uptake of [^{3}H] cholesterol by the cells was determined and corrected for total cell protein. Results are the means of duplicates which did not vary by more than 10%.

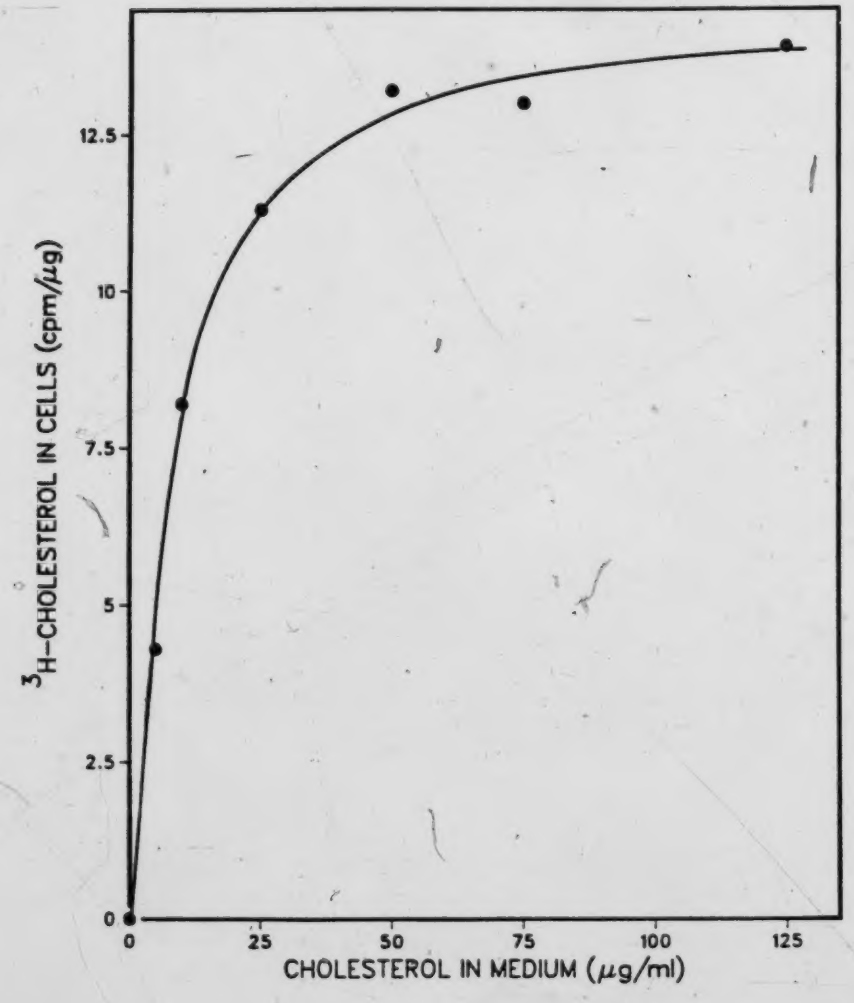


Figure 8. Effect of albumin on [^{3}H] cholesterol exchange between lipid vesicles and normal fibroblasts. Lipid vesicles (C/P = 0.6) containing tracer amounts of [^{3}H] cholesterol and [^{14}C] cholesteryl oleate were incubated at a concentration of 50 μg cholesterol/ml with fibroblast monolayers at 37°C for various times in the presence of different concentrations of bovine serum albumin. The cell monolayers were extensively washed and their content of radioactivity determined. Results are the means of duplicates which did not vary by more than 15% and are corrected for total cell protein.

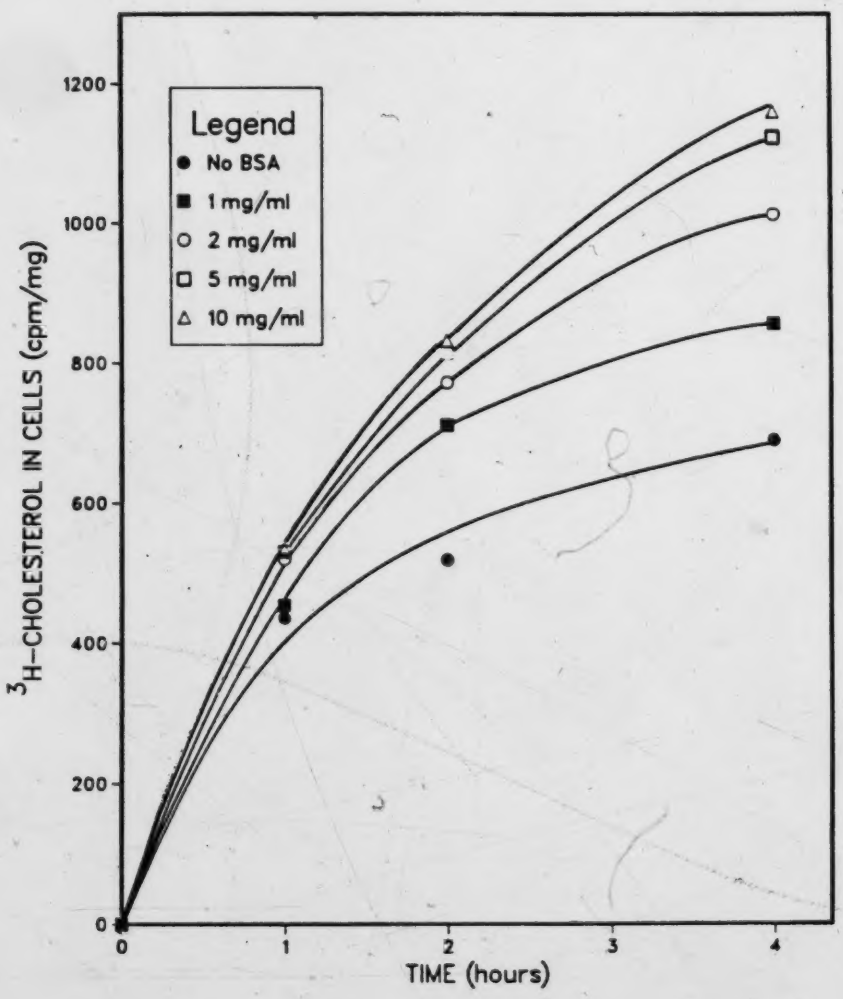


Table 4. Estimation of the amount of vesicle lipid 'sticking' to fibroblast cells. Various egg PC/cholesterol vesicles were prepared containing tracer amounts of ^{3}H-dioleoyl PC and ^{14}C-cholesteryl oleate and then incubated (0.5 mg/ml of PC) with either normal or FH fibroblasts for 6 or 24 hours. The cells were extensively washed and their content of radioactivity determined. Viability of normal fibroblasts was also determined with 0.5% nigrosin staining. Results are the means of duplicate experiments.

Vesicles added		% of radioactivity added associated with cells				% normal cell viability
		FH cells		Normal cells		
		^{14}C-CE	^{3}H-PC	^{14}C-CE	^{3}H-PC	
egg PC	6 hours	0.045	0.133	0.069	0.094	96
	24 hours	0.072	0.073	0.112	0.196	93
C/P = 0.6	6 hours	0.044	0.060	0.095	0.113	98
	24 hours	0.068	0.113	0.098	0.173	95
C/P = 2	6 hours	0.050	0.079	0.075	0.101	96
	24 hours	0.070	0.198	0.092	0.225	96

of this vesicle marker was associated with the cells (Table 4). Other control experiments measured the exchange of [^{3}H]dioleoyl PC between vesicles and normal or FH fibroblasts (Table 4). Although PC uptake by cells was greater than uptake of the cholesteryl ester, it was still less than 0.5% of the total phohspholipid added. Other experiments showed that phospholipid exchange between vesicles and fibroblast plasma membranes was very limited, even in the presence of a nonspecific lipid transfer protein purified from rat liver (Poorthuis *et al.*, 1981).

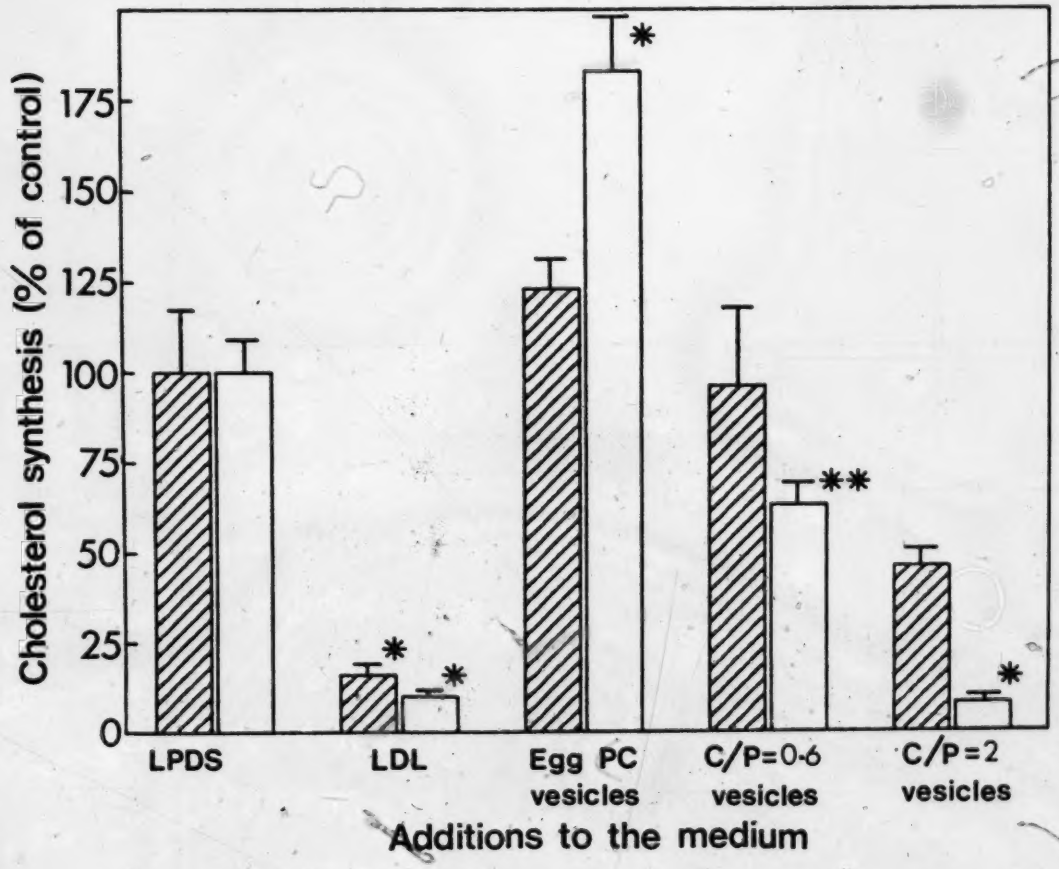
Table 5 illustrates the effects of the various incubation media on the cholesterol content of the fibroblasts. The results are similar whether expressed as cholesterol per mg protein or as a cholesterol/phospholipid (C/P) molar ratio. The stock cells cultured in 10% fetal bovine serum had an average of 31.7 μg cholesterol per mg cell protein, corresponding to a C/P of 0.421. The 24 hour preincubation of the cells in MEM containing 10% LPDS significantly reduced these values to 26.3 μg cholesterol per mg protein with C/P = 0.316. Adding 80 μg/ml LDL back to the cells resulted in a rapid increase in cell cholesterol after 6 hours which returned the cells to a similar cholesterol content as when grown in whole serum. Incubation of cells with egg PC vesicles had no significant effect on fibroblast cholesterol content after 6 hours but after 24 hours the cell cholesterol was significantly reduced. Lipid vesicles composed of cholesterol and egg PC in a C/P = 0.6 ratio had no significant effect on fibroblast cholesterol content, even after a 24 hour incubation. However, incubations of cells with cholesterol rich (C/P = 2) vesicles rapidly increased the cell cholesterol to about 32 μg/mg protein, a value similar to that for fibroblasts grown with whole serum or incubated in a medium containing LDL.

Figure 9 demonstrates the effect of the incubation with various media on the cholesterol synthesis activity of the fibroblasts. The results are expressed as a percentage of the activity of the control cells incubated in LPDS. When LDL was added back to the cells, cholesterol synthesis declined rapidly to 15% of the control value after 6 hours. This rapid down-regulation of cholesterol synthesis by LDL was no doubt due to the receptor dependent pathway described by Brown *et al.* (1974). When egg PC vesicles were incubated with the cells,

Table 5. Cholesterol content of fibroblasts following incubation with media of various compositions. Fibroblasts were grown in 10% fetal calf serum as described in the Materials and Methods section and then preincubated for 24 hours in medium containing 10% LPDS. The medium was then removed and replaced with medium containing either 80 µg of LDL/ml or 0.5 mg of lipid vesicles/ml composed of egg phosphatidylcholine or cholesterol and egg phosphatidylcholine with a C/P of 0.6 or 2. The cells were incubated at 37°C for 6 or 24 hours before determinations were made. Values are means ± S.E.M. with the number of experiments indicated in parentheses: *significantly different from control (LPDS), $P < 0.01$; †significantly different from control (LPDS), $P < 0.05$.

Addition to the medium		Cholesterol content (µg/mg of protein		C/P	
Fetal calf serum		31.7 ± 0.9*	(7)	0.421 ± 0.028*	(7)
LPDS		26.3 ± 0.4	(10)	0.316 ± 0.006	(10)
LDL	6 hours	37.8 ± 2.1*	(4)	0.414 ± 0.015*	(5)
	24 hours	30.1 ± 0.4†	(4)	0.318 ± 0.007	(5)
Egg phosphatidylcholine	6 hours	27.1 ± 1.2	(4)	0.314 ± 0.006	(4)
	24 hours	21.3 ± 1.3*	(4)	0.220 ± 0.019*	(4)
C/P = 0.6 vesicles	6 hours	28.3	(2)	0.326 ± 0.052	(4)
	24 hours	28.0 ± 0.4	(4)	0.316 ± 0.013	(5)
C/P = 2 vesicles	6 hours	33.0	(2)	0.364 ± 0.016	(3)
	24 hours	31.7 ± 1.3*	(4)	0.367 ± 0.012	(5)

Figure 9. Effects of various incubation media on cholesterol synthesis. The fibroblasts were cultured as described in the Materials and Methods section and then preincubated for 24 hours in MEM containing 10% LPDS. The medium was then removed and replaced with MEM containing either 80 μg of LDL/ml (LDL), 0.5 mg of egg phosphatidylcholine vesicles/ml (egg PC vesicles), 0.5 mg of cholesterol/egg phosphatidylcholine vesicles/ml with C/P = 0.6 (C/P = 0.6 vesicles); or 0.5 mg of cholesterol/egg phosphatidylcholine vesicles/ml with C/P = 2 (C/P = 2 vesicles). The cells were incubated for either 6 hours (hatched bars) or 24 hours (open bars) at 37°C before cholesterol synthesis activity was determined. Results are expressed as a percentage of control (LPDS) synthesis, ± S.E.M. Results are from four to six experiments. Significant difference from control: $^{*}P < 0.01$, $^{**}P < 0.05$.

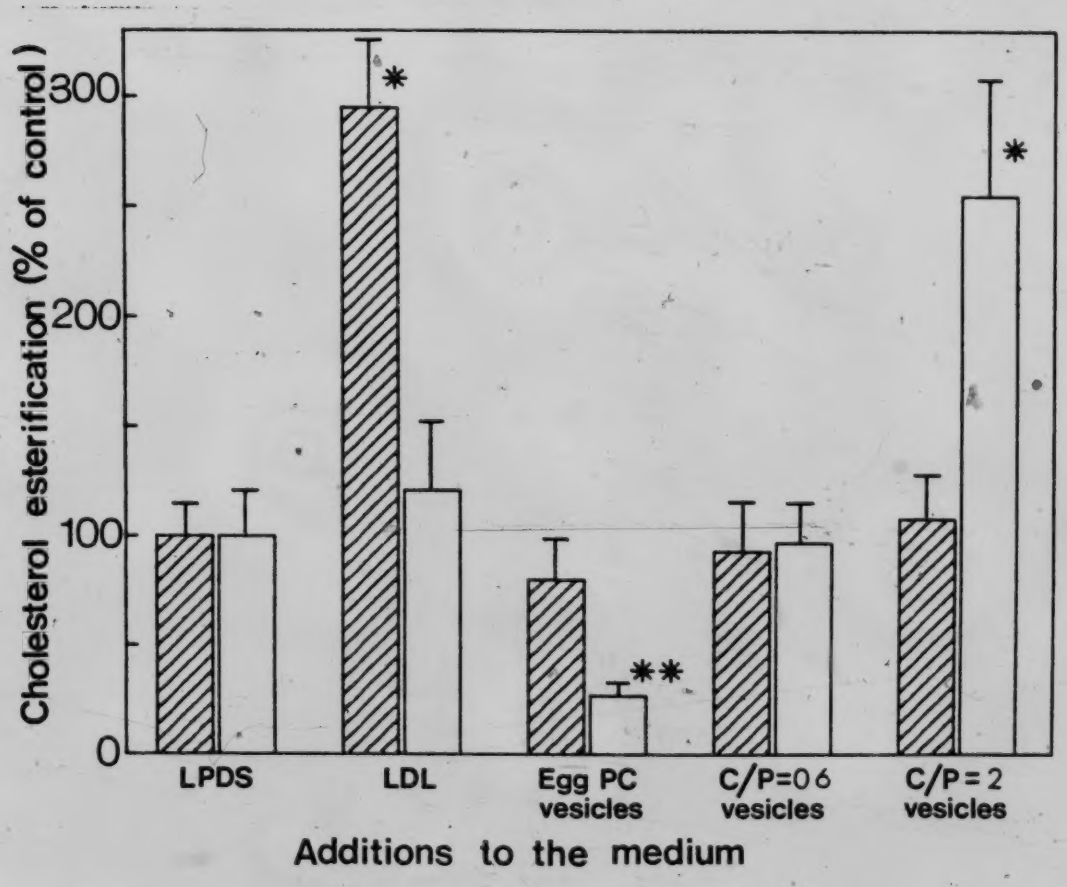


cholesterol synthesis increased slightly after 6 hours and then increased to almost twice the control value after 24 hours. Under the conditions of this incubation, we have shown a decrease in the fibroblast cholesterol content (Table 5) which we believe occurred as a result of a net efflux of cholesterol from the cell plasma membrane to the egg PC vesicles. We interpret the stimulation of endogenous cholesterol synthesis to be consequence of this efflux and the resulting decrease in cell cholesterol content. Incubation of the fibroblasts with vesicles containing cholesterol in a C/P = 0.6 ratio resulted in a small but significant decrease in cholesterol synthesis after 24 hours to about 65% of control values. In the case of the cholesterol rich (C/P = 2) vesicles, the synthesis activity was significantly reduced to less than 10% of the control after 24 hours. Under these experimental conditions we expect that cholesterol is delivered to the fibroblasts via exchange from the vesicle to the plasma membrane. This resulted in the increase in cell cholesterol shown in Table 5, and it appears that these cells respond by decreasing their endogenous cholesterol production. Using a similar protocol, Slotte and Lundberg (1983*b*) have also demonstrated decreased cholesterol synthesis in rat aortic smooth muscle cells. The fact that the C/P = 0.6 vesicles showed some limited effect on cholesterol synthesis probably indicates that these vesicles may also have some ability to deliver cholesterol to the cells.

3.2.3 Effect on cholesterol esterification

Figure 10 demonstrates the effects of the incubation of cells with either LDL or vesicles on the cholesterol esterification activity of the fibroblasts. When LDL was added to the cells, esterification activity (measured as the incorporation of ^{14}C-oleate into cholesteryl ester) was increased 3-fold after 6 hours but had returned after 24 hours to values not significantly different from control. This has been previously demonstrated for the receptor-mediated LDL pathway (Goldstein *et al.*, 1974). After 6 hours, none of the different vesicles showed any significant effect on the fibroblast esterification activity. But after 24 hours of incubation, the various vesicles had exerted significant effects which appear to be directly related to the

Figure 10. Effects of various incubation media on cholesterol esterification. The experimental conditions were as described in Figure 9. Following the incubations for 6 hours (hatched bars) or 24 hours (open bars), cholesterol esterification activity was determined. Results are expressed as a percentage of control (LPDS) incorporation of [^{3}H] oleate into cholesteryl esters, ± S.E.M. Results are from six to eight experiments, except for LDL at 6 hours (three experiments) and 24 hours (two experiments). Significant difference from control: $^*P < 0.01$, $^{**}P < 0.05$.



cholesterol content of the vesicles. The egg PC vesicles decreased esterification activity, the C/P = 0.6 vesicles had no effect, and the cholesterol rich C/P = 2 vesicles markedly stimulated the esterification activity by 2.5-fold. These data suggest that cholesterol delivered in net amounts to the plasma membrane can within 24 hours stimulate the cholesterol esterification machinery of the cell.

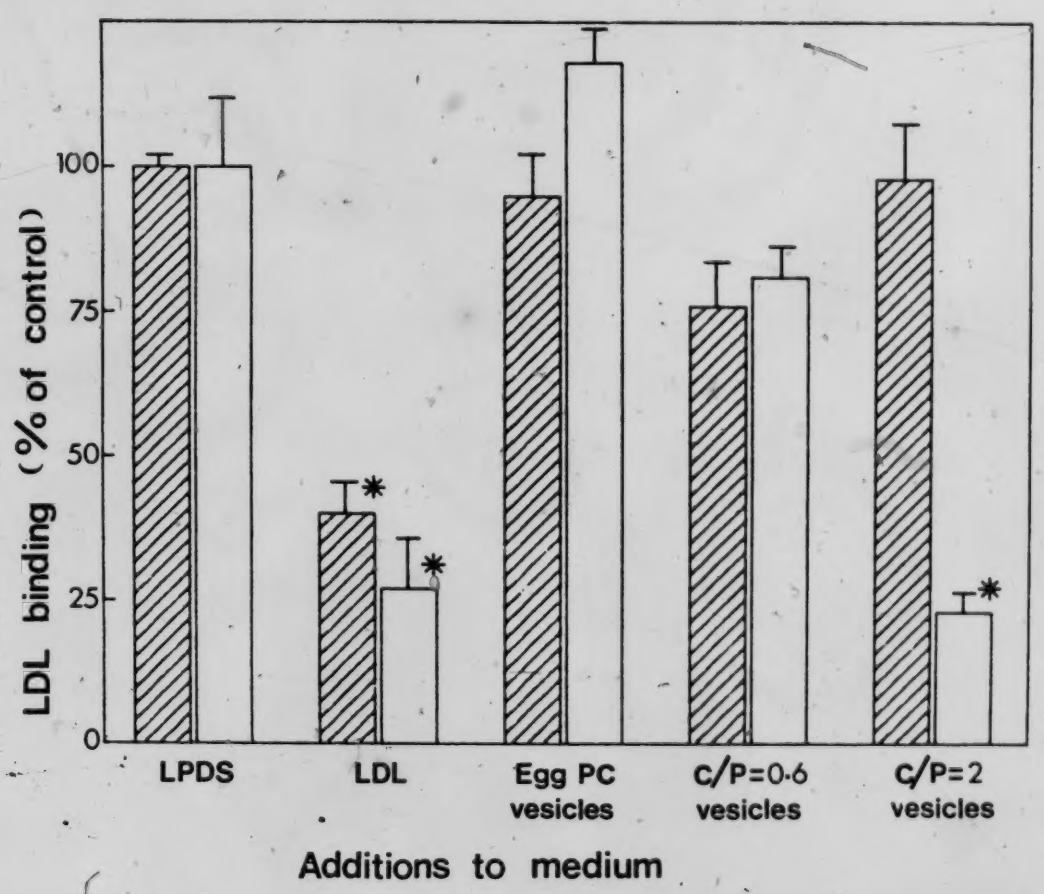
3.2.4 Effect on ^{125}I-LDL binding

Figure 11 shows the effects of the incubation with different media on the binding of ^{125}I-LDL to the fibroblasts. The average LDL binding (following the standard 24 hour incubation of cells in LPDS) was 118 ng/mg cell protein, in agreement with literature values (Brown and Goldstein, 1975). When LDL was added back to cells, we observed typical down-regulation of the LDL receptors toward basal levels by 24 hours. Incubation of cells for 6 hours with any of the various lipid vesicles had no significant effect on the binding of LDL. However, after 24 hours of incubation we observed a significant decrease in ^{125}I-LDL binding in cells exposed to cholesterol rich vesicles to levels that were similar to those obtained for cells that were incubated with LDL. Cells exposed to C/P = 0.6 vesicles showed a small but not significant decrease in LDL binding after 24 hours. Fibroblasts incubated with egg PC vesicles showed small but not statistically significant increases in LDL binding.

3.2.5 Experiments with familial hypercholesterolemia fibroblasts

A set of experiments similar to those described above were carried out to determine the effects of non-receptor mediated movements of cholesterol on the cholesterol homeostasis of FH fibroblasts. These cells lack functional LDL receptors and must therefore satisfy their cholesterol requirements by either *de novo* synthesis or by non-receptor mediated uptake of sterol from the medium. It would therefore be interesting to see how these cells modulate their intracellular cholesterol metabolism in response to sterol influx or efflux occurring at the cell surface membrane.

Figure 11. Effects of various incubation media on LDL binding. Experimental incubation conditions are described in Figure 9. Following the incubations at 37°C for 6 hours (hatched bars) or 24 hours (open bars), the fibroblast monolayers were washed and incubated with ^{125}I-LDL at 4°C for 2 hours. Specific binding was determined as described in the Materials and Methods section. Results are expressed as a percentage of control (LPDS) specific LDL binding, ± S.E.M. Results are from four to six experiments, except for egg phosphatidylcholine vesicles at 6 hours (three experiments) and for LDL at 24 hours (nine experiments). Significant different from control: $^{*}P < 0.01$.



Normal and FH fibroblasts were incubated in medium containing LPDS for 24 hours, and then exposed to the various experimental media for a further 24 hours, after which determinations of cholesterol synthesis, cholesterol esterification, and ^{125}I-LDL binding were made.

Table 6 shows the effects of the experimental incubations on cellular cholesterol synthesis. For the normal fibroblasts, typical effects were observed with FCS, LDL, and the C/P = 2 vesicles markedly decreasing cholesterol synthesis while the egg PC vesicles significantly stimulated biosynthesis (compare to Figure 9). As observed in other experiments, the C/P = 0.6 vesicles also showed some ability to partially down regulate cholesterol synthesis, in this case by some 30%.

The FH fibroblasts responded in a qualitatively similar fashion to the various incubation media, with decreased cholesterol synthesis occurring in cultures which received FCS, LDL, or C/P = 2 vesicles and significantly increased synthesis in the presence of egg PC vesicles. In all cases the magnitude of the response appeared to be less in the FH cells as compared to the normal fibroblasts.

The effects of the different media on normal and FH fibroblast cholesterol esterification are presented in Table 7. Again the results for normal cells are in agreement with previous experiments (see Figure 10), with a particularly striking six-fold stimulation of esterification by the C/P = 2 vesicles. Medium containing FCS also stimulated cholesterol esterification, but to a lesser extent.

The results for the FH fibroblasts are for the most part qualitatively similar to those obtained for normal cells. A very large stimulation was observed for C/P = 2 vesicles. Interestingly, a three-fold stimulation of esterification occurred in the presence of LDL, which differs from the situation with normal cells, where no stimulation was observed after 24 hours. This result is consistent with the idea that LDL cholesterol which enters the FH cell by exchange at the plasma membrane takes much longer to stimulate the cellular cholesterol esterification machinery than does LDL cholesterol which enters normal cells via the LDL receptor pathway.

Table 6. Effects of various incubation media on cholesterol synthesis in normal and FH fibroblast cells. The experimental conditions were as described in Figure 9, except the incubation was for 24 hours. Results are expressed as a percentage of control (LPDS) synthesis ± 1 S.D. with the number of samples indicated in parentheses. *significantly different from control, $P < 0.01$.

Addition to medium	Normal cells		FH Cells	
LPDS	100%	(4)	100%	(4)
FCS	12 ± 4	(3)*	45 ± 2	(2)*
LDL	8 ± 1	(3)*	34 ± 2	(3)*
Egg PC vesicles	145 ± 22	(4)*	116 ± 9	(3)*
C/P = 0.6 vesicles	68 ± 9	(3)*	43 ± 31	(4)*
C/P = 2 vesicles	8 ± 1	(4)*	30 ± 4	(3)*

Table 7. Effects of various incubation media on cholesterol esterification in normal and FH fibroblasts. The experimental conditions were as described in Table 6. Results are expressed as a percentage of control (LPDS) esterification ± 1 S.E. with the number of samples indicated in parentheses. *significantly different from control, $p < 0.01$.

Addition to medium	Normal cells	FH cells
LPDS	100 (4)	100 (4)
FCS	295 ± 72 (3)*	145 ± 85 (3)
LDL	69 ± 91 (3)	317 ± 5 (2)*
Egg PC vesicles	15 ± 8 (3)*	88 ± 16 (4)
C/P = 0.6 vesicles	106 ± 14 (3)	189 ± 60 (4)
C/P = 2 vesicles	641 ± 75 (2)*	1288 ± 265 (4)*

In a final series of experiments, the effects of the different incubation media on the binding of ^{125}I-LDL to FH fibroblasts was determined. As anticipated, no significant effect was observed and all FH cell cultures showed only background ^{125}I-LDL binding equal to the non-specific binding (data not shown).

3.3 Uptake and Cellular Processing of LDL-Associated Phosphatidylcholine

In order to further investigate the different kinds of lipid movements between serum and cultured fibroblasts, it was decided to examine a receptor mediated process, namely the receptor mediated endocytosis of LDL and its associated lipids. LDL has four major components which together constitute over 90% of the particle by weight (Lee, 1976; Krieger *et al.*, 1978): 1) apolipoprotein B (22%), cholesteryl esters (38%), phospholipids (23%), and free cholesterol (8%). The protein component can be radiolabelled with ^{125}I as described above in section 3.1. However, introduction of radioactive lipids into the LDL particle has generally required more complex extraction/reconstitution procedures. A sodium deoxycholate reconstitution method has been described (Ginsburg *et al.*, 1982; Walsh and Atkinson, 1983; Ginsburg *et al.*, 1984) but in our hands the apoprotein/lipid recombinant particle contained sufficient deoxycholate as to be injurious to cells. Instead the heptane extraction procedure of Krieger *et al.* (1978, 1979) has been used to produce recombinant LDL particles. This method was originally used to introduce exogenous cholesteryl esters into LDL. In the present studies this method was modified to allow incorporation of exogenous phospholipid into the recombinant particle as well. In addition, exogenous free cholesterol could be introduced into the reconstituted LDL by a modification of the method of Lundberg *et al.* (1982). Overall this resulted in the capability to selectively radiolabel all of the four major LDL components listed above.

3.3.1 Preparation and characterization of reconstituted LDL particles

In the first series of experiments, LDL was reconstituted with exogenous [^{3}H]cholesteryl linoleate according to the procedure of Krieger *et al.* (1978) to produce a soluble, reconstituted particle hereafter designated as r[^{3}H-CE]LDL. This particle was similar in chemical composition to the one described in the original method (Table 8), containing 0.84 mg cholesteryl linoleate/mg protein with no free cholesterol remaining. The extraction procedure appears to remove some of the LDL phospholipid, reducing its concentration to 0.69 mg/mg protein. The yield of protein of 48% agrees well with the results obtained by Krieger *et al.* (1978).

A second series of reconstituted LDL particles were prepared in order to determine if exogenous phospholipid could be incorporated into LDL by this procedure. In this case the reconstitution was carried out in the presence of 6 mg of non-radioactive cholesteryl linoleate and increasing amounts of [^{3}H]dioleoyl phosphatidylcholine (DOPC). The phospholipid was added in heptane solution, along with the cholesteryl linoleate as described in the original method. This produced a number of reconstituted LDL particles collectively referred to as r[^{3}H-DOPC]LDL.

The effect of increasing amounts of DOPC in the recombination mixture on the overall yield of rLDL protein was determined. As shown in Figure 12, increasing amounts of DOPC reduced the yield of r[^{3}H-DOPC]LDL protein. From 0 to 200 μgm of DOPC added, the yield declined by more than 50%. For more than 500 μgm of DOPC added the yield of protein declined to near zero, and this was accompanied by substantial turbidity in the samples.

The content of cholesteryl ester, phospholipid, and [^{3}H]DOPC radioactivity were determined for the different r[^{3}H-DOPC]LDL recombinants. These data were normalized to the amount of rLDL protein and are presented in Figure 13. As can been seen, the presence of PC in the reconstitution mixture had very little effect on the CE content of the r[^{3}H-DOPC]LDL, increasing it only by about 10% at the highest PC concentrations. The phospholipid content, as determined by phosphate assay, increased with increasing added [^{3}H]DOPC to a maximum of

Table 8. Chemical composition of reconstituted LDL. Duplicate samples of LDL (1.9 mg protein) were heptane extracted and reconstituted with 6 mg of cholesteryl linoleate. The protein, cholesteryl ester, free cholesterol, and phospholipid content of the untreated LDL and the reconstituted LDL were determined as described under Materials and Methods.

Sample	Protein	Free Cholesterol	Cholesteryl ester	Phospho-lipid
Native (untreated) LDL	1.90	0.52	1.62	1.36
Reconstituted LDL	0.91	< 0.005	0.76	0.69

Figure 12. Effect of phosphatidylcholine on the yield of reconstituted LDL protein. LDL was reconstituted as described under Materials and Methods in the presence of various amounts of [^{3}H] dioleoyl phosphatidylcholine (DOPC). The amount of solubilized protein was determined by the method of Lowry *et al* (1951). Data are the means of duplicate samples which did not vary by more than 15%.

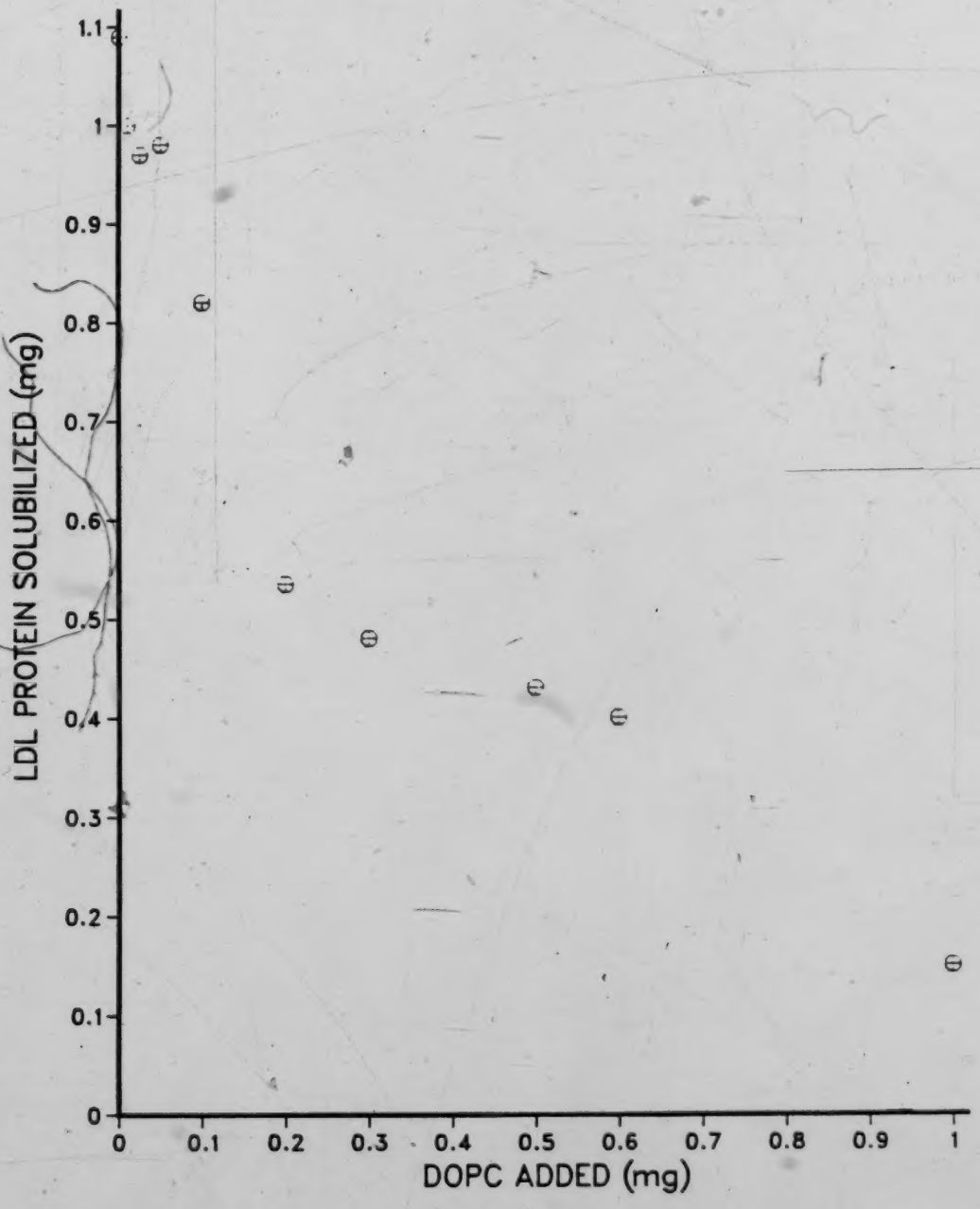
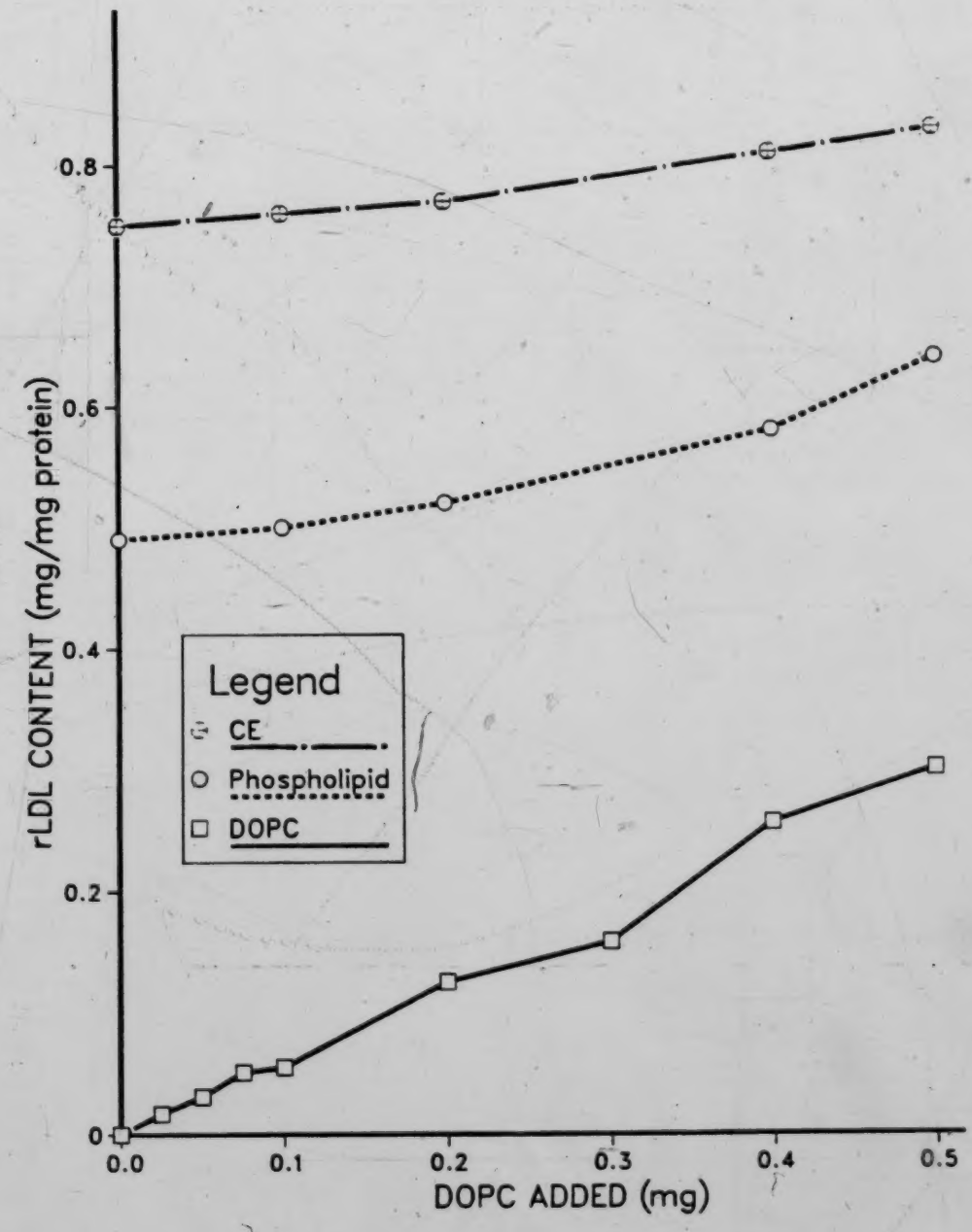


Figure 13. Effect of increasing amounts of added phosphatidylcholine on the reconstituted LDL content of cholesteryl ester (CE), phospholipid (Phospholipid) and [^{3}H] dioleoyl phosphatidylcholine ([^{3}H] DOPC). Experimental conditions were as described in Figure 12. Total cholesterol and phospholipid were determined as described under Materials and Methods. [^{3}H] DOPC content was calculated from the amount of solubilized radioactivity. Results are the means of duplicate samples.



0.64 mg/mg protein. This represents an increase of 30% compared to the rLDL prepared in the absence of exogenous phospholipid. The content of [^{3}H]DOPC, on the other hand, increased to a maximum of 0.3 mg incorporated per mg LDL protein when 0.5 mg of [^{3}H]DOPC was included in the reconstitution mixture. Taken together, these results imply that some of the [^{3}H] DOPC associated with the rLDL represents net addition of phospholipid, while the remainder most likely represents radiolabel incorporated by an exchange process. Assuming that PC represents 75% of the total LDL phospholipid before reconstitution and that the added [^{3}H] DOPC exchanges only with other PC molecules, it can be calculated that a very substantial amount (perhaps up to 90%) of the incorporated [^{3}H]DOPC represents phospholipid added to the reconstituted LDL by exchange.

This interpretation was supported by thin layer chromatography of the phospholipids of a recombinant particle prepared in the presence of 300 μg of [^{3}H]DOPC (Table 9). Even though the amount of radioactivity associated with the rLDL implied that 130 μg of [^{3}H]DOPC had been incorporated, chemical assay of the phosphatidylcholine demonstrated in fact that a small (not significant) decrease in PC content had occurred. Therefore, the net uptake of [^{3}H] DOPC observed must have been accompanied by a compensating net movement of LDL phospholipid in the opposite direction. The content of rLDL sphingomyelin also appeared to be slightly decreased, although again this result was not significantly different from the untreated LDL.

3.3.2 Fibroblast uptake and degradation of single-labelled reconstituted LDL

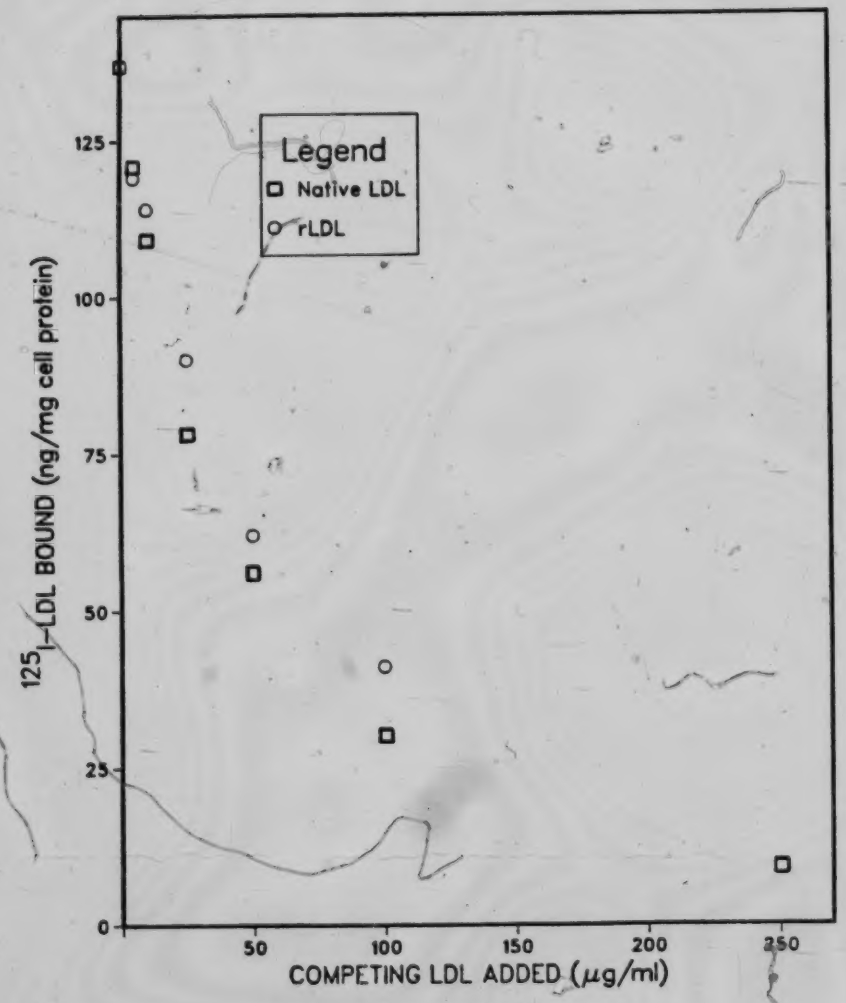
3.3.2.1 Uptake and degradation of r[^{3}H-CE]LDL

As a preliminary test for the biological activity of the reconstituted LDL particle, native LDL and r[CE]LDL were compared for their ability to compete against ^{125}I-LDL for binding to the cell surface of normal fibroblasts at 4°C. As shown in Figure 14, the reconstituted and native LDL were of almost equal potency in their ability to compete with ^{125}I-LDL for the LDL receptor.

Table 9. Phospholipid composition of r[^{3}H-DOPC]LDL. The reconstituted particle was prepared as described under Materials and Methods in the presence of 0.3 mg [^{3}H]DOPC. Duplicate lipid extracts were subjected to polar thin layer chromatography. The phospholipids were eluted from the plates and their concentrations determined as described under Materials and Methods. The amount of [^{3}H]DOPC incorporated, as calculated by the radioactivity present, equalled 0.13 mg.

	Phospholipid content (mg/mg protein)	
Phospholipid	Untreated LDL	r[^{3}H-DOPC]LDL
Phosphatidylcholine	0.50	0.48
Sphingomyelin	0.14	0.10

Figure 14. Competition curves for native or reconstituted LDL against ^{125}I-LDL binding to fibroblasts. Cells were preincubated in medium containing 10% LPDS for 24 hours before use. Monolayers received 10 μg/ml ^{125}I-LDL and various concentrations of either unlabelled native LDL (Native LDL) or r[CE]LDL (rLDL) for 4 hours at 4°C. ^{125}I-LDL binding was determined as described under Materials and Methods. Each value represents a single sample for rLDL and triplicate samples for native LDL.



r[CE]LDL was also tested for its ability to regulate intracellular cholesterol metabolism. As shown in Table 10, the r[CE]LDL showed an almost equal ability as native LDL to decrease fibroblast cholesterol synthesis and to stimulate cholesterol esterification. For both processes, the r[CE]LDL induced a cellular response that was about 80% of that occurring in the presence of an equal amount of native LDL protein. This result may be due to a partial loss of biological function of the LDL apolipoprotein during the reconstitution procedure, or may instead be simply a result of the fact that r[CE]LDL contains less total cholesterol than native LDL (Table 8).

To directly measure the metabolism of the r[^{3}H-CE]LDL it was incubated with normal fibroblasts and its cellular uptake and hydrolysis were measured as a function of time. As shown in Figure 15, the cellular content of intact ^{3}H-cholesteryl linoleate increased with time and reached a plateau within two hours. Free ^{3}H-cholesterol very rapidly appeared in the cells and continued to accumulate in a linear fashion up to four hours. By that time approximately 85% of the ^{3}H-radioactivity in the cell was in the hydrolyzed, free cholesterol form. This hydrolysis of the CE of the r[^{3}H-CE]LDL was prevented by 100 μM chloroquine, a weak base which accumulates in acidic organelles and which inhibits acid-dependent processes (Dean *et al.*, 1984). In the presence of 100 μM chloroquine the cellular accumulation of r[CE]LDL was reduced, but even more striking, the content of free versus esterified cholesterol was reversed so that after 4 hours over 80% of the ^{3}H-radioactivity was in the unhydrolyzed CE form. These results demonstrate that the r[^{3}H-CE]LDL was delivered to the fibroblast lysosome, where the CE was rapidly degraded to free cholesterol which remained in the cell. These results are in agreement with those of Krieger *et al.* (1978).

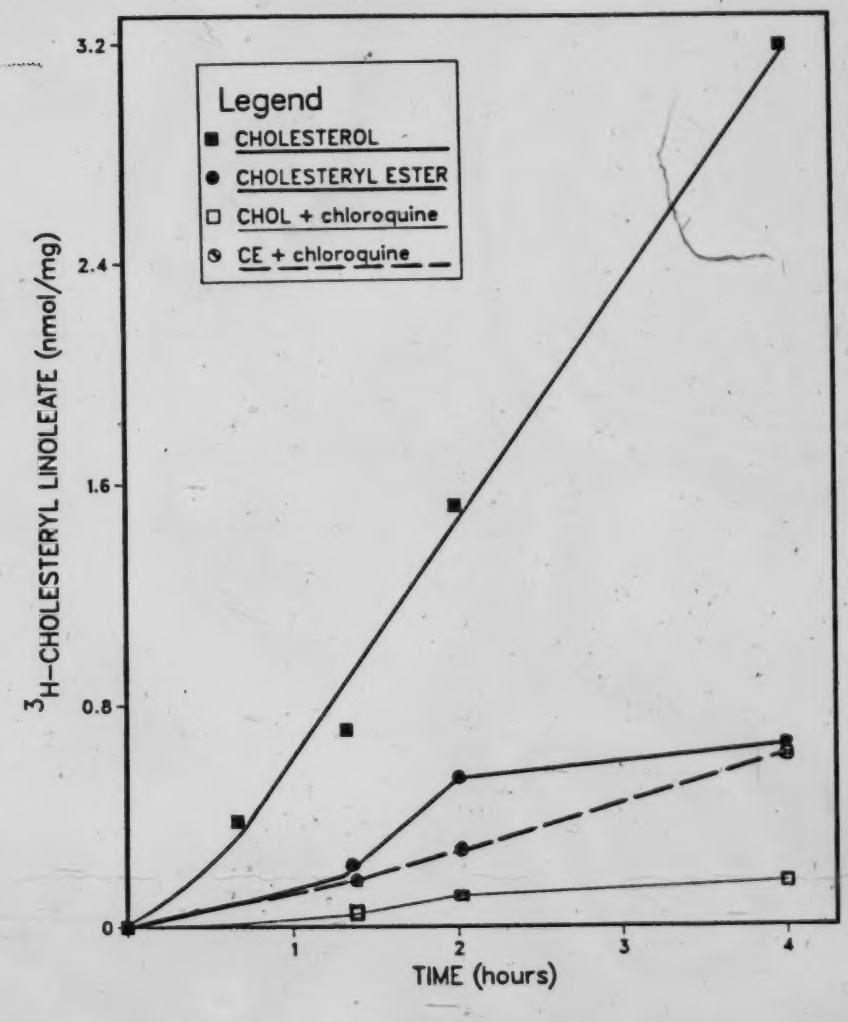
3.3.2.2 Uptake and metabolism of different reconstituted LDL particles containing exogenous phosphatidylcholines

Various reconstituted LDL particles containing radioactive phosphatidylcholines were prepared by the modified reconstitution procedure described under Materials and

Table 10. Suppression of cholesterol synthesis and stimulation of cholesterol esterification by native and reconstituted LDL. Fibroblasts were preincubated for 24 hours in medium containing 10% LPDS and were then exposed to medium containing either native LDL or r[CE]LDL (80 µg/ml) for 4 hours at 37°C in the presence of either 10 µCi of ^{14}C-acetate or 10 µCi of ^{14}C-oleic acid. Cholesterol synthesis and cholesterol esterification were determined as described under Materials and Methods.

Addition to the medium	Cholesterol synthesis (cpm acetate incorporated/mg protein)		Cholesterol esterification (cpm oleic acid incorporated/mg protein)	
Control (10% LPDS)	82,268 64,981	(100%)	41,738 52,017	(100%)
Native LDL	13,558 12,949	(18%)	173,504 152,773	(346%)
r[CE]LDL	21,389 25,242	(32%)	133,324 114,260	(264%)

Figure 15. Time course of the uptake and hydrolysis of r[^{3}H-CE]LDL in the presence and absence of chloroquine. Fibroblasts were preincubated for 24 hours in medium containing 10% LPDS. Cells then received r[^{3}H]LDL (20 μg/ml) in the presence or absence of 100 μM chloroquine and were incubated at 37°C for the indicated times. The cell lipids were extracted, the cholesterol and CE separated by tlc, and samples were counted as described under Materials and Methods. Each value represents the mean of duplicate incubations which did not vary by more than 15%, and are corrected for cell protein.



Methods. Two different preparations of r[^{3}H-DOPC]LDL were produced by including 0.3 mg of [^{3}H]DOPC (10 mCi/mmol) in the reconstitution mixture; these are referred to as r[^{3}H-DOPC 1]LDL and r[^{3}H-DOPC 2]LDL. Two other LDL recombinants, containing DPPC labelled in either the choline head group or the acyl chains, were prepared by including 0.3 mg of carrier egg PC containing 75 μCi of either [choline-methyl-^{3}H]DPPC or [2-palmitoyl-9, 10-^{3}H]DPPC in the reconstitution; these are designated r[^{3}H-choline-DPPC]LDL and r[^{3}H-palmitoyl-DPPC]LDL. The specific activities of these reconstituted LDL particles, expressed as cpm/mg LDL protein, were as follows: r[^{3}H-DOPC 1] LDL, 0.97 x 10^6 cpm/mg; r[^{3}H-DOPC 2]LDL, 1.37 x 10^6 cpm/mg; r[^{3}H-choline-DPPC] LDL, 6.78 x 10^6 cpm/mg; and r[^{3}H-palmitoyl-DPPC]LDL, 6.17 x 10^6 cpm/mg. When these reconstituted LDL particles were incubated with normal fibroblasts there was similar uptake of the PC radioactivity for each preparation (Figure 16). Fibroblasts from a familial hypercholesterolemia homozygote, which lack functional LDL receptors, took up less than 15% of the PC, as did the normal cells.

The cellular uptake of the r[^{3}H-DOPC 2]LDL was examined in more detail. A lipid extract of the rLDL was chromatographed and the concentration of PC determined to be 0.49 mg/mg protein. This corresponds to a specific activity of 2.24 x 10^6 cpm/μmol of rLDL PC. The uptake of this particle by normal fibroblasts grown in medium containing LPDS or FCS, or by FH fibroblasts, was then determined. As shown in Figure 17, cells incubated in 10% LPDS took up approximately 3.5 nmol of [^{3}H]DOPC in 4 hours. This is a reasonable rate of PC uptake, compared to the uptake of CE by normal fibroblasts (Krieger *et al.*, 1978). Normal fibroblasts which had been grown in whole serum, which suppresses LDL receptor activity, showed very little uptake ($<$ 15% of the value in LPDS) of rLDL associated PC. FH fibroblasts did not take up the [^{3}H]DOPC to any significant extent. This result suggests that there is very little exchange of the DOPC with the fibroblast cell membrane, and that virtually all the radioactivity associated with the normal cells most likely resulted from receptor mediated uptake of the r[^{3}H-DOPC]LDL.

Figure 16. Time course of the cellular uptake of various LDL particles reconstituted with exogenous phosphatidylcholines. The cells were preincubated in medium containing 10% LPDS for 24 hours to induce maximal LDL receptor activity. Reconstituted LDL particles (20 μg/ml) containing either [^{3}H]DOPC (DOPC 1 or DOPC 2) or [^{3}H-choline]DPPC (DPPC 1) or [^{3}Hpalmitoyl]DPPC (DPPC 2) were incubated for the times shown. The monolayers were washed and the content of cellular radioactivity and protein determined as described under Materials and Methods. Data are the means of either duplicate or triplicate samples which did not vary by more than 15%.

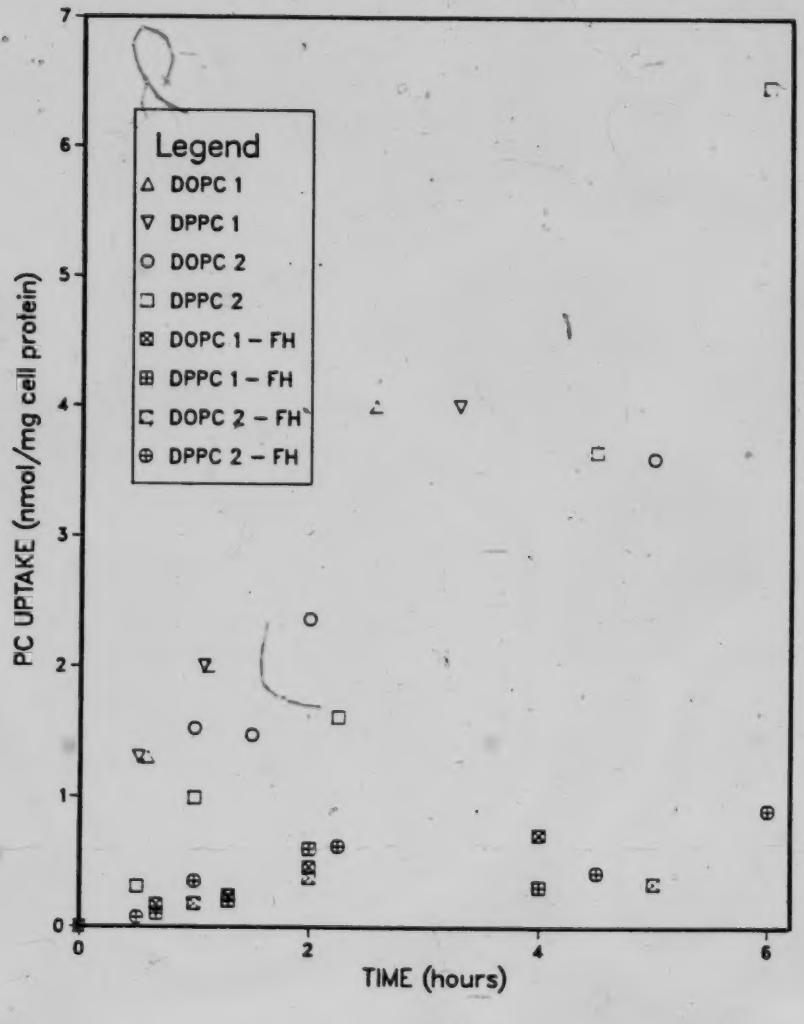
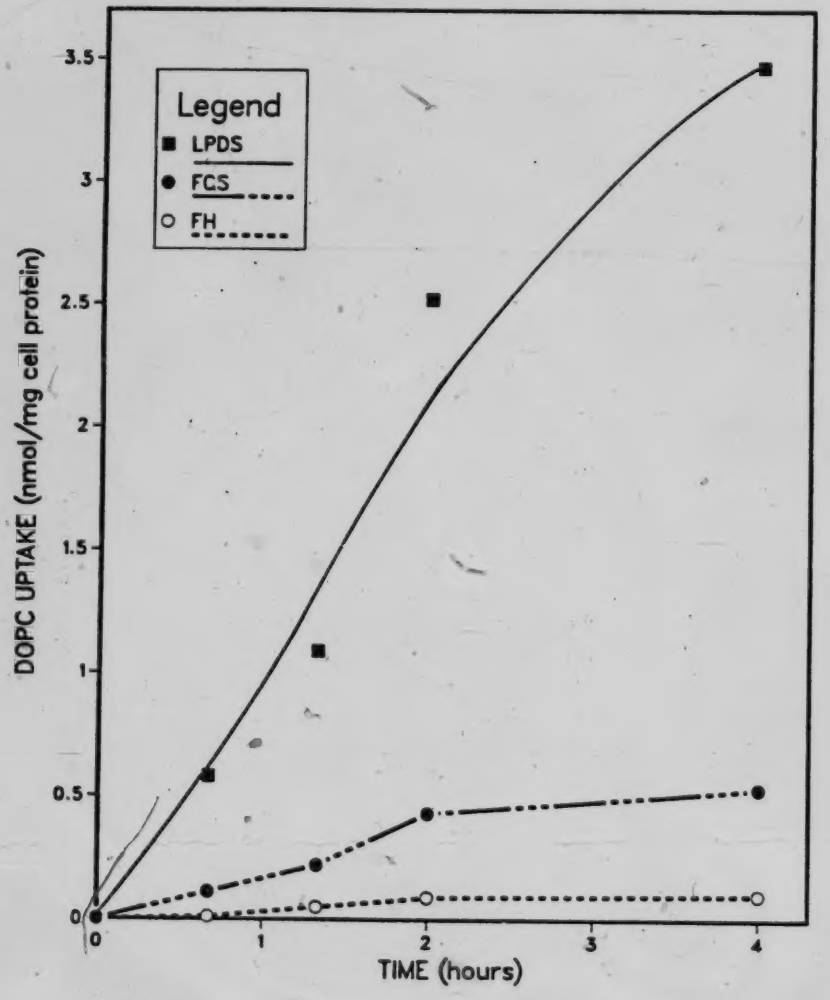


Figure 17. Uptake of LDL associated DOPC by normal and FH fibroblasts. Normal cells were preincubated for 24 hours in LPDS (LPDS) or FCS (FCS). FH cells were preincubated for 24 hours in LPDS (FH). r[^{3}H-DOPC]LDL (20 μg/ml) was added to the monolayers for various times and cellular uptake of radioactivity determined as described under Materials and Methods. Results are the means of duplicates which did not vary by more than 10%.



When the cellular lipids of normal fibroblasts which had been exposed to any of the radioactive PC-containing rLDLs were examined, an interesting observation was made. It had been shown that these LDL recombinants were recognized by the LDL receptor and it was assumed that they were subsequently delivered to the lysosomes for hydrolysis, as was shown for the CE component of r[^{3}H-CE]LDL. However, thin layer chromatography of the cellular lipids consistently showed that the radiolabelled PC remained essentially intact throughout the time course of the experiment and that 80-95% of the cellular radioactivity could be recovered in the PC spot on the TLC plates. An example of a scan of the radioactivity present on the TLC plates is presented in Figure 18. The radioactive PC accumulated in the cell in a time dependent manner, with no evidence of breakdown or conversion to other lipids. When the TLC plates were stained, cut into segments and counted by liquid scintillation counting, again virtually all of the radioactivity was associated with the PC spot (Table 11).

These results suggested several possibilities: 1) that the r[PC]LDL particles were bound to the cell but for some reason were not internalized; 2) that the r[PC]LDL was delivered intact to the lysosomes, but the PC was for some reason resistant to hydrolysis; or 3) the r[PC]LDL was delivered to lysosomes, but at some point along the endocytic pathway a substantial amount of its associated PC segregated from the particle, perhaps via exchange with endogenous phospholipid, and thus escaped lysosomal hydrolysis.

That at least a part of the rLDL PC reached the lysosomes was suggested by results of experiments in which r[^{3}H-choline-DPPC]LDL was incubated with normal and FH fibroblasts in the presence or absence of 100 μM chloroquine. The uptake of the rLDL was similar for normal fibroblasts in the presence or absence of chloroquine for up to 4.5 hours (Figure 19) after which the chloroquine appeared to inhibit further LDL uptake. FH cells did not take up the r[^{3}H-choline-DPPC]LDL to any significant extent. When the lipids were extracted from the normal and FH cells and chromatographed on TLC plates, once again essentially all of the radioactivity appeared in the PC spot (greater than 80% for all

Figure 18. Radioactivity scan of thin layer chromatogram of lipids extracted from fibroblasts incubated with r[DPPC]LDL. r[^{3}H-palmitoyl-DPPC]LDL was incubated (20 μg/ml) with normal fibroblasts (which had been preincubated in 10% LPDS for 24 hours) for various times. The monolayers were washed, and the cellular lipids were extracted and chromatographed as described under Materials and Methods. The plates were scanned with a Bioscan radioactivity scanner.

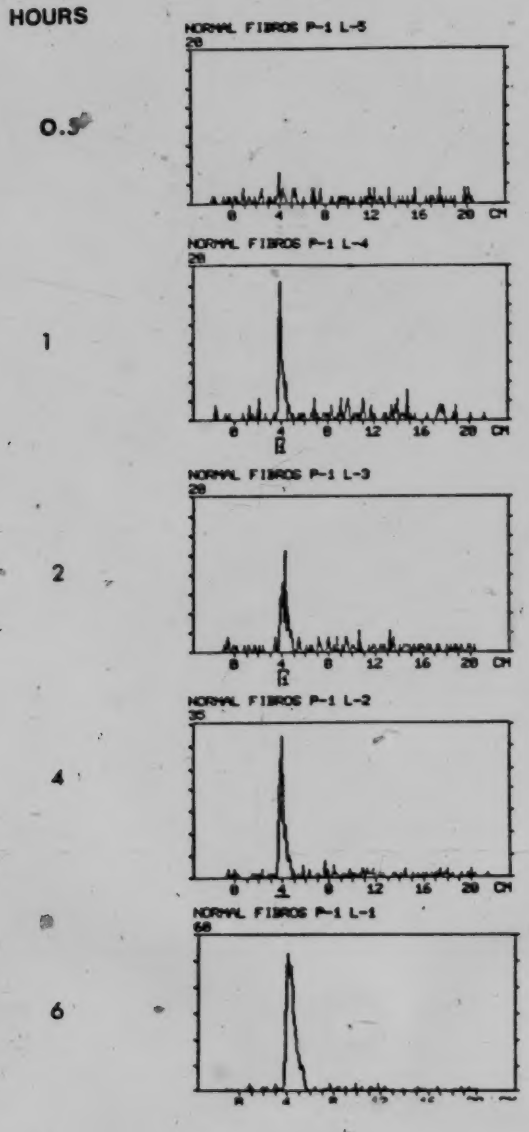
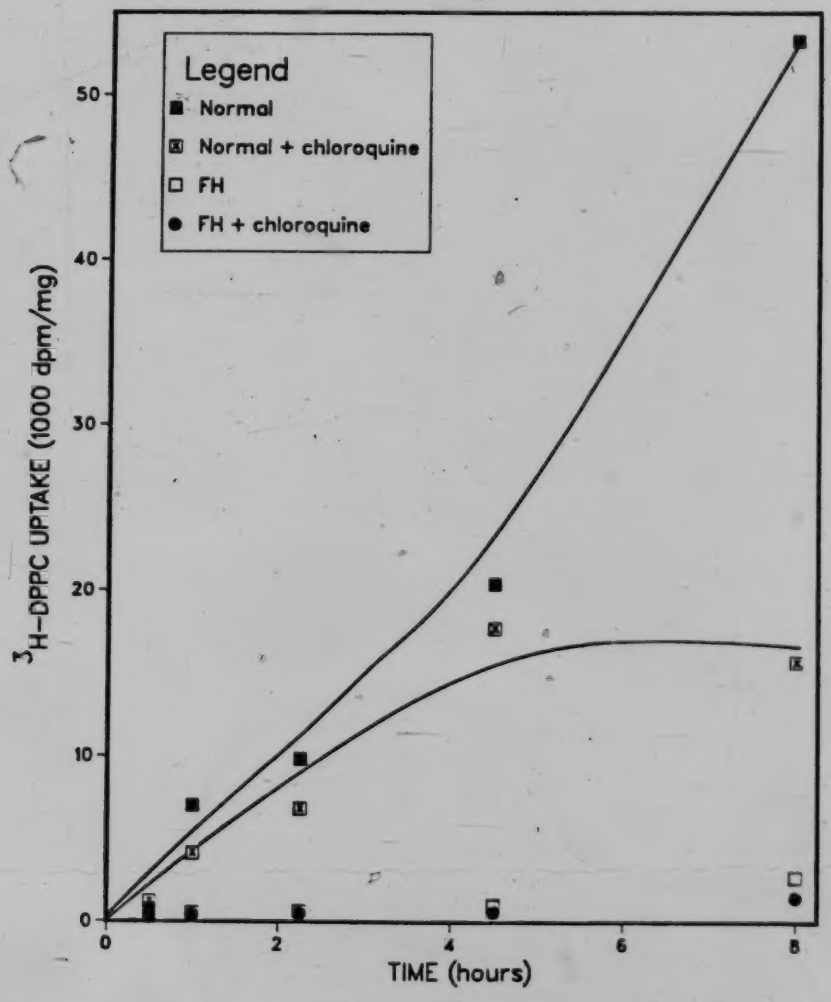


Table 11. Amount of fibroblast radioactivity associated with the PC spot on tlc plates following incubation of cells with rLDLs containing radioactive PC. Reconstituted LDL particles were incubated at 20 µg/ml with normal fibroblasts which had been preincubated in 10% LPDS for 24 hours. After various times, the monolayers were washed and the cellular lipids were extracted, chromatographed, and counted as described under Materials and Methods. Results are the means of duplicates which did not vary by more than 20%.

	% of radioactivity associated with PC	
Time (hours)	r[^{3}H-DOPC]LDL	r[^{3}H-palmitoyl]LDL
0.5	-	83
1	91	90
2	94	89
4	90	93
6	89	91

Figure 19. Time course of uptake of r[^{3}H-choline-DPPC]rLDL by normal and FH fibroblasts. Cells were treated as in Figure 17, except that 20 μg/ml r[^{3}H-choline-DPPC] was incubated with the monolayers in the presence or absence of 100 μM chloroquine. Results are from single samples and are corrected for total cell protein.



time points and incubation conditions). However, when the aqueous layer from each lipid extract was counted, it revealed a time dependent, chloroquine-inhibitable appearance of radioactivity (Figure 20). It should be recalled that for the first four hours of the incubation, approximately equal amounts of radioactivity was taken up by the normal cells in the presence of 100 μM chloroquine as in its absence (Figure 19). These results suggest that at least a portion of the LDL associated DPPC choline head group was converted by the fibroblast to a water soluble compound by an acid-dependent process. This evidence is most consistent with the second or third possibilities discussed above, namely that although some of the LDL associated phospholipid reaches the lysosome and is degraded to smaller, water soluble molecules, the majority of the PC remains resistant to hydrolysis. This could either be due to exchange of the radioactive PC with cellular lipid prior to the arrival of the lipoprotein at the lysosome, or to some unexplained resistance of DPPC to lysosomal phospholipases.

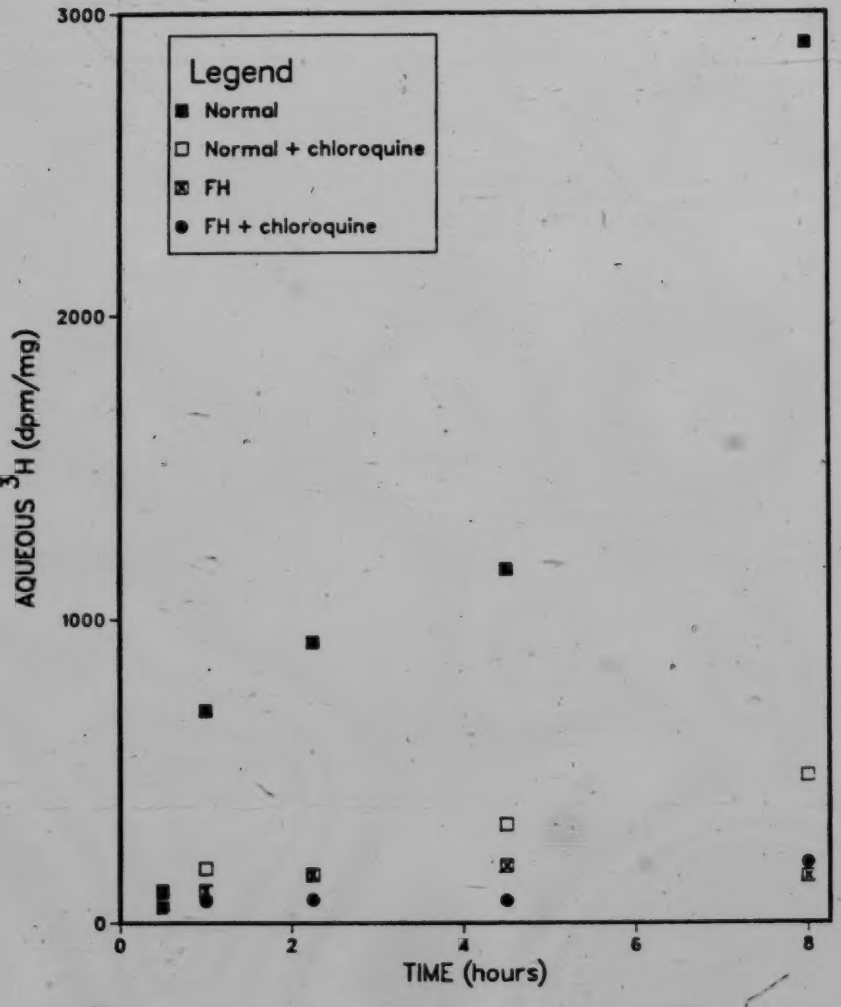
3.3.3 Uptake and Cellular Processing of Double-Labelled Reconstituted LDL

In light of of the preceding results, it was decided to further investigate the uptake and metabolism of LDL-associated PC. In order to determine the possible mechanisms by which rLDL PC apparently escaped lysosomal hydrolysis, reconstituted LDL particles were prepared containing two separate radiolabels in either the protein or lipid components. In this manner it was possible to directly compare the uptake and cellular metabolism of PC with that of the other LDL components.

3.3.3.1 Cellular processing of rLDL labelled in PC and CE

The first double-labelled rLDL was prepared by the usual reconstitution procedure in the presence of 150 μCi of [^{3}H]cholesteryl linoleate (labelled in the cholesterol) and 10 μCi of [^{14}C]DOPC (labelled in the fatty acyl chains). The reconstituted particle, designated r[^{3}H-CE/^{14}C-DOPC]LDL, had a specific activity for the CE of 1.58 x 10^6 cpm ^{3}H/mg protein and for PC the specific activity equalled 0.35 x 10^6 cpm ^{14}C/mg protein. This

Figure 20. Time course of the appearance of ^{3}H-radioactivity in the aqueous layer of a lipid extract of normal and FH fibroblasts incubated with r[^{3}H-choline-DPPC]LDL in the presence or absence of 100 μM chloroquine. Experimental conditions as described in Figure 19.



corresponds to a $^{3}H/^{14}C$ ratio of 4.53. When the r[^{3}H-CE/^{14}C-DOPC]LDL was incubated with normal fibroblasts for up to 5 hours, both ^{3}H and ^{14}C radioactivity accumulated in the cells in a time dependent manner (Figure 21). With increasing time, the $^{3}H/^{14}C$ ratio inside the cell appeared to increase gradually (Table 12). At the earliest time points the ratio of ^{3}H to ^{14}C was the same in the cells as for the intact r[^{3}H-CE/^{14}C-DOPC]LDL. This suggests that both the PC and CE were taken up by the cell together. At later time points the $^{3}H/^{14}C$ ratio increased by about 20% after 4 or 5 hours. It is not entirely clear why this ratio should increase. One possibility, assuming that at least a portion of the internalized PC is catabolized (as suggested by the data in Figures 19 and 20), is that a part of any [^{14}C]oleic acid released by cellular phospholipases may leak out of the cell into the culture medium. This would lead to the increase in $^{3}H/^{14}C$ ratio observed. However, attempts to demonstrate an accumulation of radioactive free fatty acids in the medium were unsuccessful. The medium lipids were extracted and subjected to neutral thin layer chromatography. There was no significant difference in the amount of radioactivity in the fatty acid spot from medium which had been incubated with normal fibroblasts compared to that of FH cells, nor did it increase with time. There is therefore no direct evidence for the hydrolysis of the [^{14}C]DOPC.

The cellular lipids of the normal fibroblasts which had been incubated with the r[^{3}H-CE/^{14}C-DOPC]LDL were extracted and the neutral and polar lipids separated by TLC. Hydrolysis of the [^{3}H]CE to free cholesterol took place very rapidly, as shown in Figure 22. Within 0.5 hours of incubation [^{3}H]cholesterol appeared in the cells and it increased in an essentially linear manner for 5 hours. The [^{3}H]CE in the cell reached a plateau after 0.5 hours that was maintained until 5 hours. These results are similar to those obtained with the single-labelled r[^{3}H-CE]LDL shown previously in Figure 15. However, the [^{14}C]DOPC component of the double-labelled rLDL did not appear to be substantially hydrolyzed ($<$ 18% at all time points) and accumulated in the cell approximately in parallel with the [^{3}H]free cholesterol (Figure 22). The similar shape of these two curves

Figure 21. Time course of the cellular uptake of r[^{3}H-CE/^{14}C-DOPC]LDL. Fibroblasts were preincubated in medium containing 10% LPDS for 24 hours. Monolayers were incubated with 20 μg/ml r[^{3}H-CE/^{14}C-DOPC]LDL at 37°C for the indicated times. Data are the means of duplicate samples that varied by less than 15%.

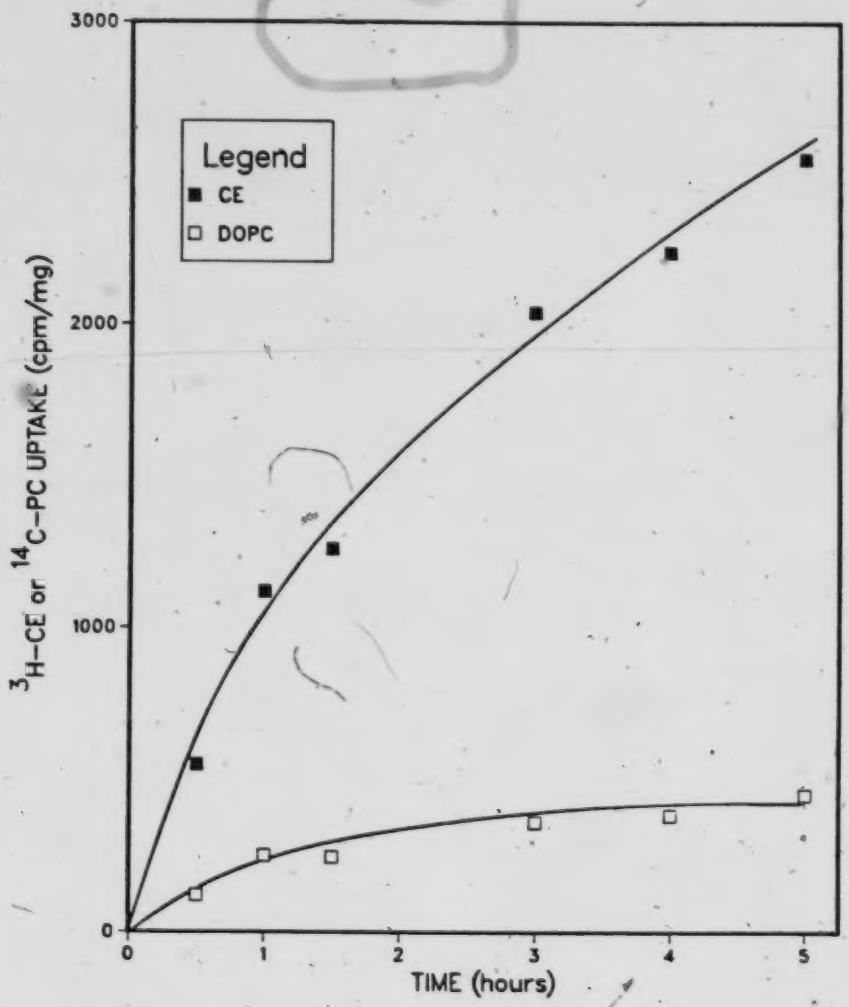
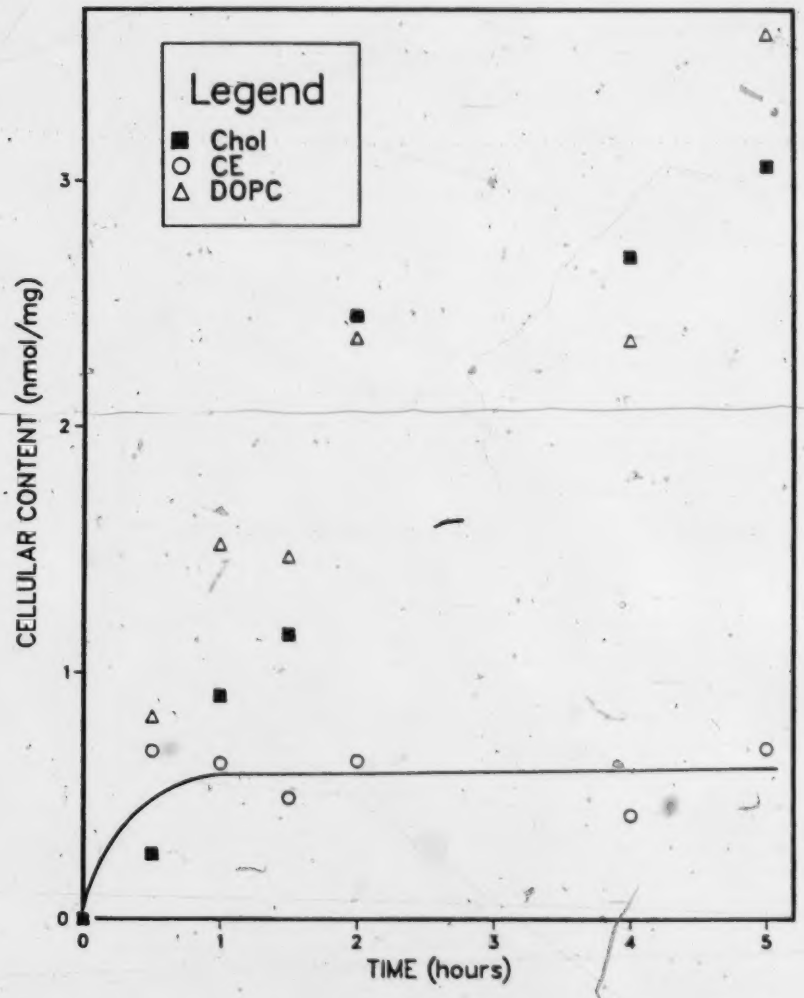


Table 12. $^{3}H/^{14}C$ ratio of fibroblasts following incubation with r[^{3}H-CE/^{14}C-DOPC]LDL. Experimental procedures were as described in Figure 21. $^{3}H/^{14}C$ ratio of the rLDL was 4.53.

Time (hours)	$^{3}H/^{14}C$
0.1	4.58
1	4.48
1.5	5.25
2	5.60
4	5.89
5	5.67

Figure 22. Time course of the hydrolysis of the CE but not the DOPC of r[^{3}H-CE/^{14}C-DOPC] LDL. Experimental conditions were as described in Figure 21 for normal fibroblasts. The cellular lipids were extracted and chromatographed by neutral and polar tlc as described under Materials and Methods. The amount of radioactivity present in the free cholesterol (^{3}H-cholesterol), cholesteryl esters (^{3}H-CE) and DOPC (^{14}C-DOPC) was determined. Results are the means of duplicate samples which did not vary by more than 20%.



also suggests that significant breakdown of the DOPC did not occur.

3.3.3.2 Cellular processing of r[^{125}I/^{3}H-DOPC]LDL

To directly compare the uptake and metabolism of the apoprotein and phospholipid components of LDL, a reconstituted LDL particle was prepared from ^{125}I-LDL in the presence of 0.3 mg [^{3}H]DOPC. This r[^{125}I/^{3}H-DOPC]LDL had a specific activity of 304 cpm ^{125}I/ng protein. The specific activity of the ^{3}H-DOPC was determined by lipid extraction of an aliquot of the rLDL followed by polar TLC and liquid scintillation counting of the PC spot. This was necessary to reduce the interference caused by any residual traces of ^{125}I-radioactivity when trying to count the [^{3}H]DOPC.

The r[^{125}I/^{3}H-DOPC]LDL was incubated (50 μg/ml) with normal fibroblasts which had been preincubated in medium containing 10% LPDS. The time course of the cellular uptake of [^{3}H]DOPC and ^{125}I-LDL is shown in Figure 23. Total uptake of both the protein and the phospholipid was similar. Thin layer chromatography of lipids extracted from the cells showed that greater than 75% of the ^{3}H radioactivity associated with the cells appeared in the PC spot, consistent with only partial hydrolysis of the LDL-associated DOPC. The hydrolysis of the apoprotein component of r[^{125}I/^{3}H]LDL was determined and was compared to that of the "native" ^{125}I-LDL (Figure 24). In both cases, the cellular content of ^{125}I-labelled protein was in a steady state plateau after 2 hours. This represents LDL which had been taken up by the fibroblasts but which had not yet been hydrolyzed in the lysosomes. ^{125}I-LDL which had been hydrolyzed to its component amino acids was excreted by the cells into the medium as trichloroacetic acid soluble material (upper curves of Figure 24). ^{125}I-radioactivity from r[^{125}I/^{3}H-DOPC]LDL was released from the fibroblasts at a similar rate to that from "native" ^{125}I-LDL. This indicates that the apoprotein component of the double-labelled, reconstituted LDL was delivered to the lysosomes and hydrolyzed. Since the phospholipid component of this reconstituted LDL was taken up by the cells (Figure 23) but not catabolized (as shown by TLC), it appears that the PC component of r[^{125}I/^{3}H-DOPC]LDL escapes lysosomal hydrolysis.

Figure 23. Time course of the uptake of r[^{125}I/^{3}H-DOPC]LDL. Fibroblasts were preincubated in 10% LPDS for 24 hours before use. Monolayers were incubated for various times with 50 μg/ml of r[^{125}I/^{3}H-DOPC]LDL. The cells and medium were treated as described under Materials and Methods. Total cellular uptake of ^{125}I-LDL (LDL) was calculated as described under Materials and Methods. [^{3}H]DOPC uptake (DOPC) was determined by counting of the PC spot following tlc. Results are the means of duplicate incubations and did not vary by more than 20%.

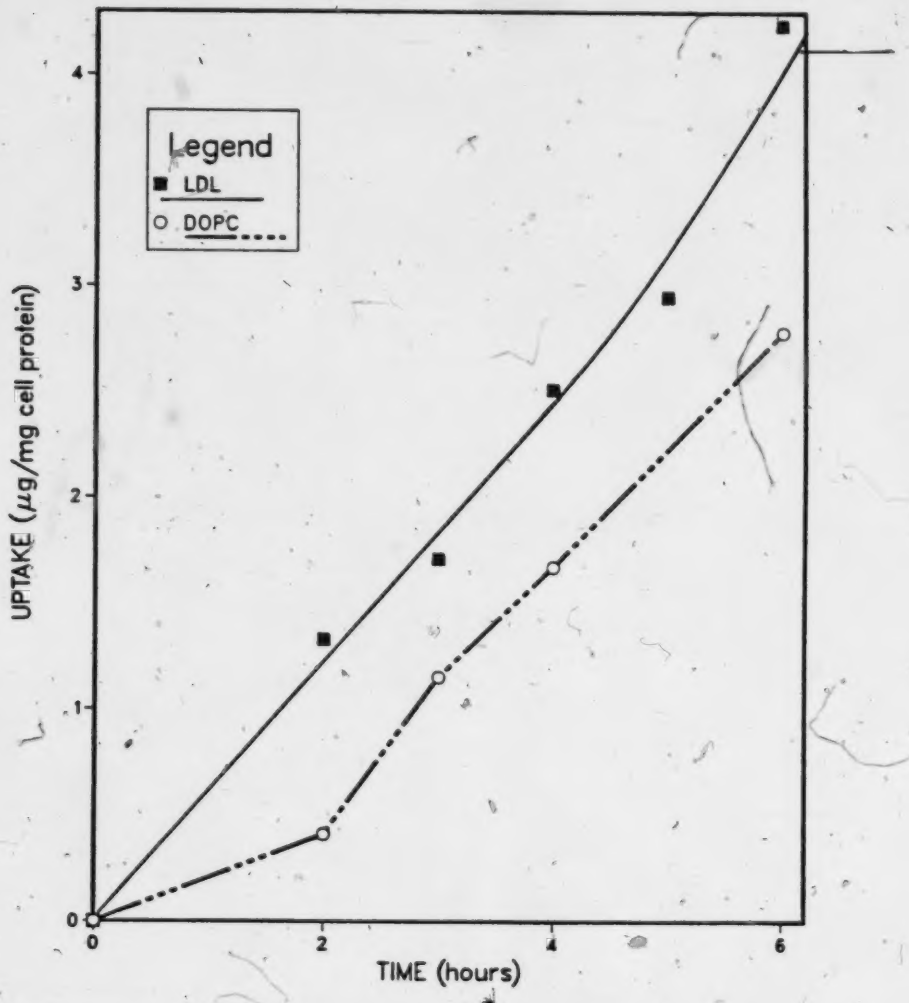
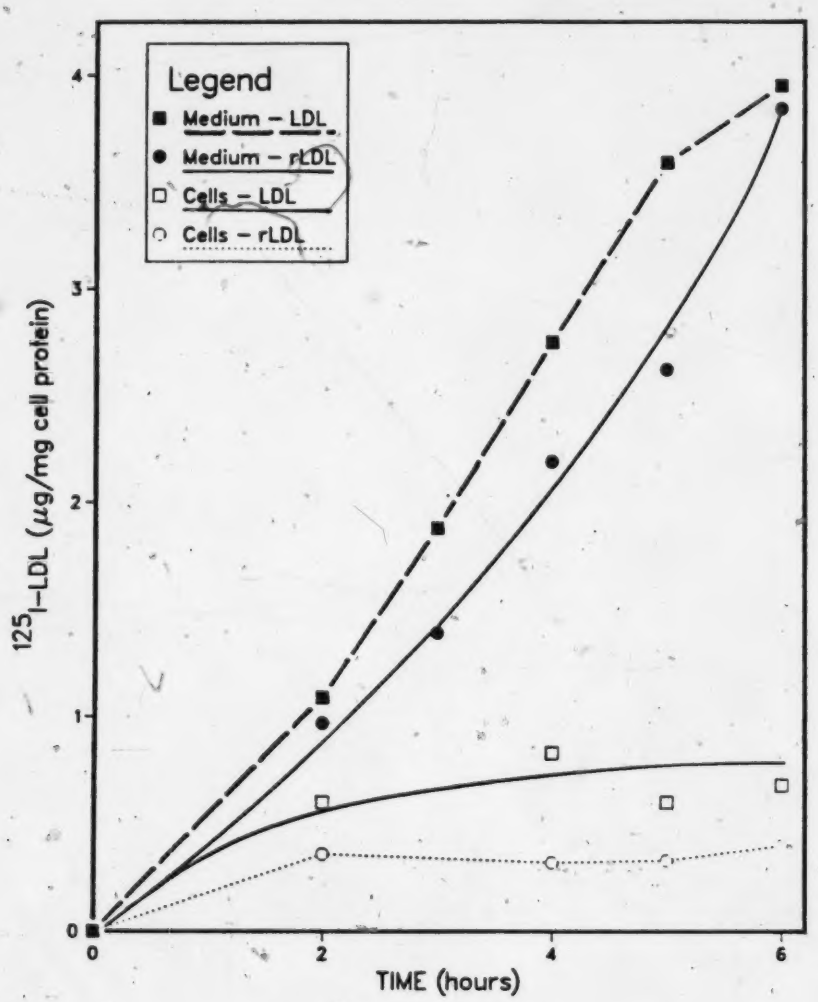


Figure 24. Time course of the uptake and cellular hydrolysis of ^{125}I-LDL and r[^{125}I/^{3}H-DOPC]LDL. Experimental conditions were as described in Figure 23. Monolayers received 50 μg/ml of either ^{125}I-LDL or r[^{125}I/^{3}H-DOPC]LDL for the indicated times. The total cellular content of ^{125}I-LDL (Cells-LDL) and r[^{125}I/^{3}H-DOPC]LDL (Cells-rLDL) were determined as described under Materials and Methods. The total amount of ^{125}I-protein hydrolyzed and excreted into the medium for ^{125}I-LDL (Medium-LDL) and r[^{125}I/^{3}H-DOPC]LDL (Medium-rLDL) were determined as described under Materials and Methods. Each value represents the mean of duplicate samples.



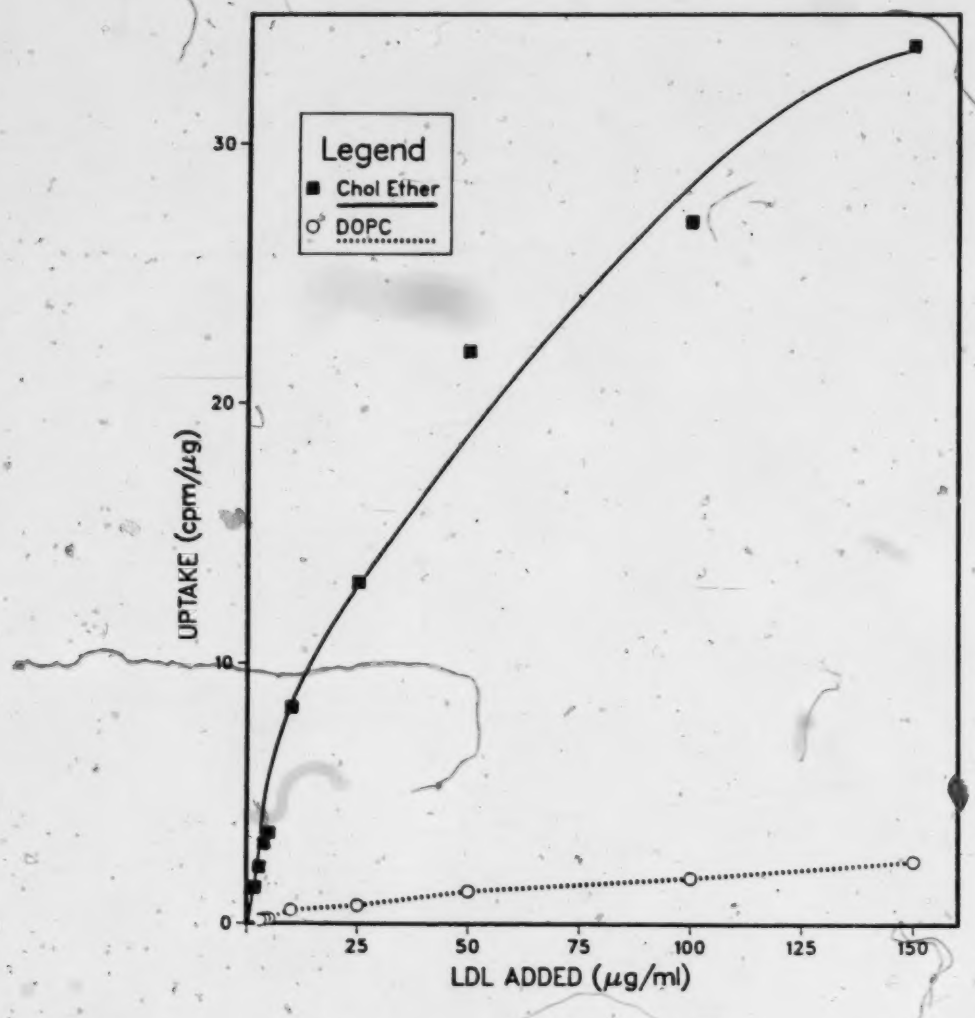
3.3.3.3 Cellular uptake of rLDL containing labelled PC and cholesteryl hexadecyl ether

To directly compare the cellular uptake and processing of LDL-associated phospholipid with another LDL component which is known not to be catabolized, a double-labelled reconstituted LDL particle was prepared containing tracer amounts of [^{14}C]DOPC and [^{3}H]cholesteryl hexadecyl ether. This ether analogue of cholesteryl ester is resistant to esterase hydrolysis and has been used previously in the study of lipoprotein metabolism (Higgs *et al.*, 1984). The rLDL was prepared in the usual manner except that 250 μCi of [^{3}H]cholesteryl hexadecyl ether was included with the unlabelled cholesteryl linoleate. The reconstituted particle, designated r[^{3}H-CEther/^{14}C-DOPC]LDL, had a specific activity of 2.34 x 10^{6} ^{3}H-cpm and 0.16 x 10^{6} ^{14}C-cpm per mg protein. When the rLDL was incubated with fibroblasts, both the DOPC and cholesteryl ether components were taken up in a concentration dependent manner (Figure 25). The ^{3}H/^{14}C ratio for the two components was the same in the cells as for the rLDL and remained essentially constant for all concentrations. Neither label was taken up by FH cells to any appreciable extent. The similar shape of the uptake curves for the [^{14}C]DOPC and [^{3}H]cholesteryl ether suggests that the PC, like the ether analogue of CE, remains unhydrolyzed by the cells.

3.3.3.4 Estimation of the recycling of LDL-associated DPPC

The results of the preceding experiments, involving the incubation of single and double-labelled rLDL particles with normal and FH fibroblasts, demonstrated that LDL-associated PC substantially escaped lysosomal hydrolysis and remained intact within the cell. One possibility which had not been investigated was that perhaps the PC was in fact catabolized, but that the breakdown products were reutilized in the synthesis of new PC molecules. In order to test this possibility, an experiment was carried out with an rLDL containing DPPC radiolabelled in the choline head group. This r[^{3}H-DPPC]LDL was prepared with 0.3 mg carrier egg PC and 75 μCi [choline-methyl-^{3}H]DPPC, and had a specific activity of 5.6 x 10^{4} cpm/mg protein. The rLDL was incubated with normal fibroblast cells at a concentration of 20 μg/ml at 37°C for various times up to 7 hours. A lipid

Figure 25. Concentration dependence of the uptake of r[^{3}H-CEther/^{14}C-DOPC]LDL by normal fibroblasts. Cells were preincubated in medium containing 10% LPDS for 24 hours before use, and then were incubated with various concentrations of r[^{3}H-CEther/^{14}C-DOPC]LDL for 3 hours at 37°C. Uptake was determined as described under Materials and Methods. Results are the means of duplicate incubations.



extract of the cells showed a time dependent increase in radioactivity in both the aqueous and organic layers of the extract (Figure 26). The radioactivity in the aqueous layer represented a constant fraction of that in the organic layer at each time, averaging 5% of the total. This was as shown previously for another preparation of r[^{3}H-choline-DPPC]LDL, when an average of 8% of the total radioactivity appeared in the aqueous layer of a lipid extract of cells which had been incubated with the rLDL (see Figure 19 and Figure 20). These results again suggest that a fraction of the internalized PC has been hydrolyzed by the cells.

In order to determine if the LDL-associated [^{3}H]DPPC was being hydrolyzed by the cells with subsequent reutilization of the radiolabelled headgroup for new PC synthesis, it was necessary to separate the dipalmitoyl PC species from the other PC molecules present in the cell. To accomplish this, a polar TLC procedure was used in which half of the lipid extracts were treated with osmium tetroxide as described under Materials and Methods. This procedure resulted in the retention at the origin of most of the PC molecules containing unsaturated fatty acids, while the DPPC species appeared in the regular PC spot (Table 13).

When this procedure was applied to the lipids extracted from fibroblasts which had been incubated with r[choline-^{3}H-DPPC]LDL, the results demonstrated that only a small amount of cell associated radioactivity remained at the TLC plate origin (Figure 27). This amounted to about 25% of the total radioactivity after 7 hours of incubation. In other words, three quarters of the [^{3}H]DPPC associated with the cell after 7 hours remained in the dipalmitoyl form. This result again demonstrates the substantial, although not complete, resistance to cellular hydrolysis of PC taken up by the fibroblast via the LDL receptor pathway.

Figure 26. Time course of the cellular uptake of r[choline-^{3}H-DPPC] radioactivity into the organic and aqueous layers of a lipid extract of normal fibroblasts. Cells were preincubated for 24 hours in medium containing 10% LPDS and were then exposed for various times to 20 μg/ml of r[choline-^{3}H-DPPC]LDL. The cell lipids were extracted and counted as described under Materials and Methods. Results are the means of duplicate incubations which did not vary by more than 15%.

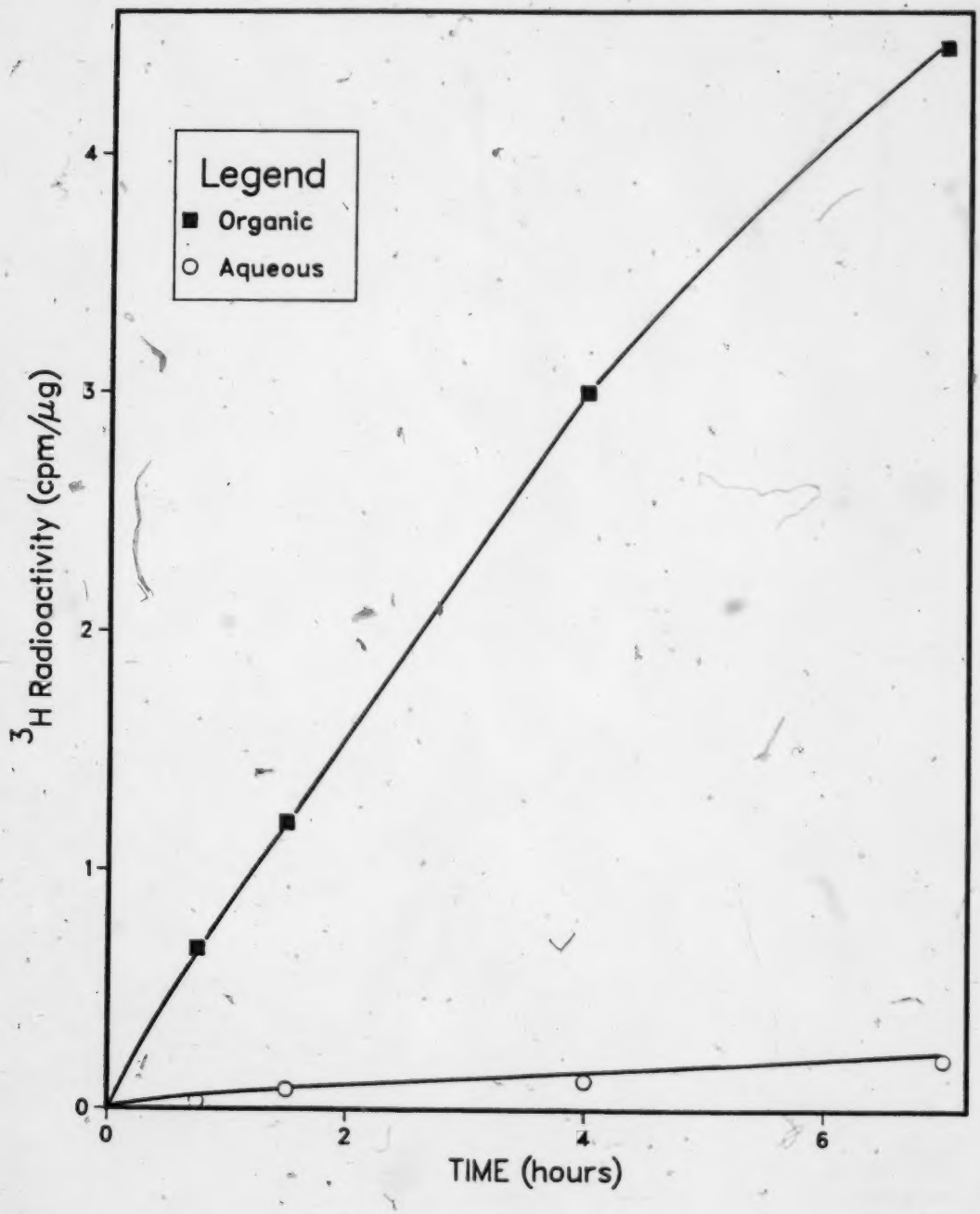
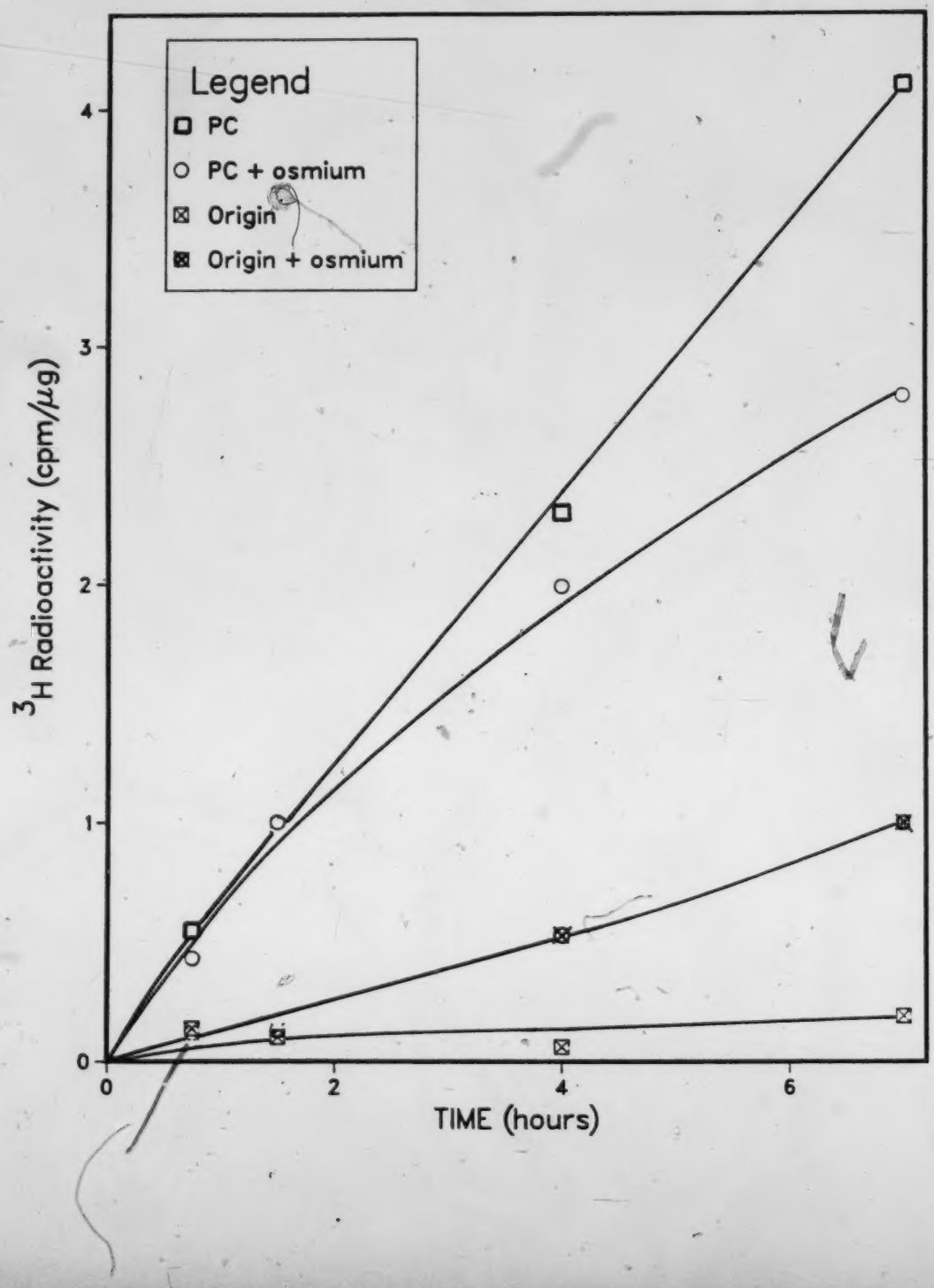


Table 13. Quantitation of the separation of DPPC from other PC species by tlc of osmium tetroxide-treated lipid extracts. Samples of egg PC containing tracer amounts of [^{3}H]DOPC or [^{3}H]DPPC were treated as described under Materials and Methods. Some of the samples were treated with osmium tetroxide before tlc. Following polar tlc the amount of radioactivity was determined at the origin and in the PC spot. Results are the means ± 1 S.D. for four samples.

		% of cpm	
^{3}H-lipid	Treatment	Origin	PC
DOPC	-	6.8 ± 1.5	93.2 ± 1.5
DOPC	+ osmium	91.3 ± 4.8	8.6 ± 3.1
DPPC	-	4.5 ± 1.0	95.4 ± 0.9
DPPC	+ osmium	21.4 ± 1.0	78.6 ± 1.0

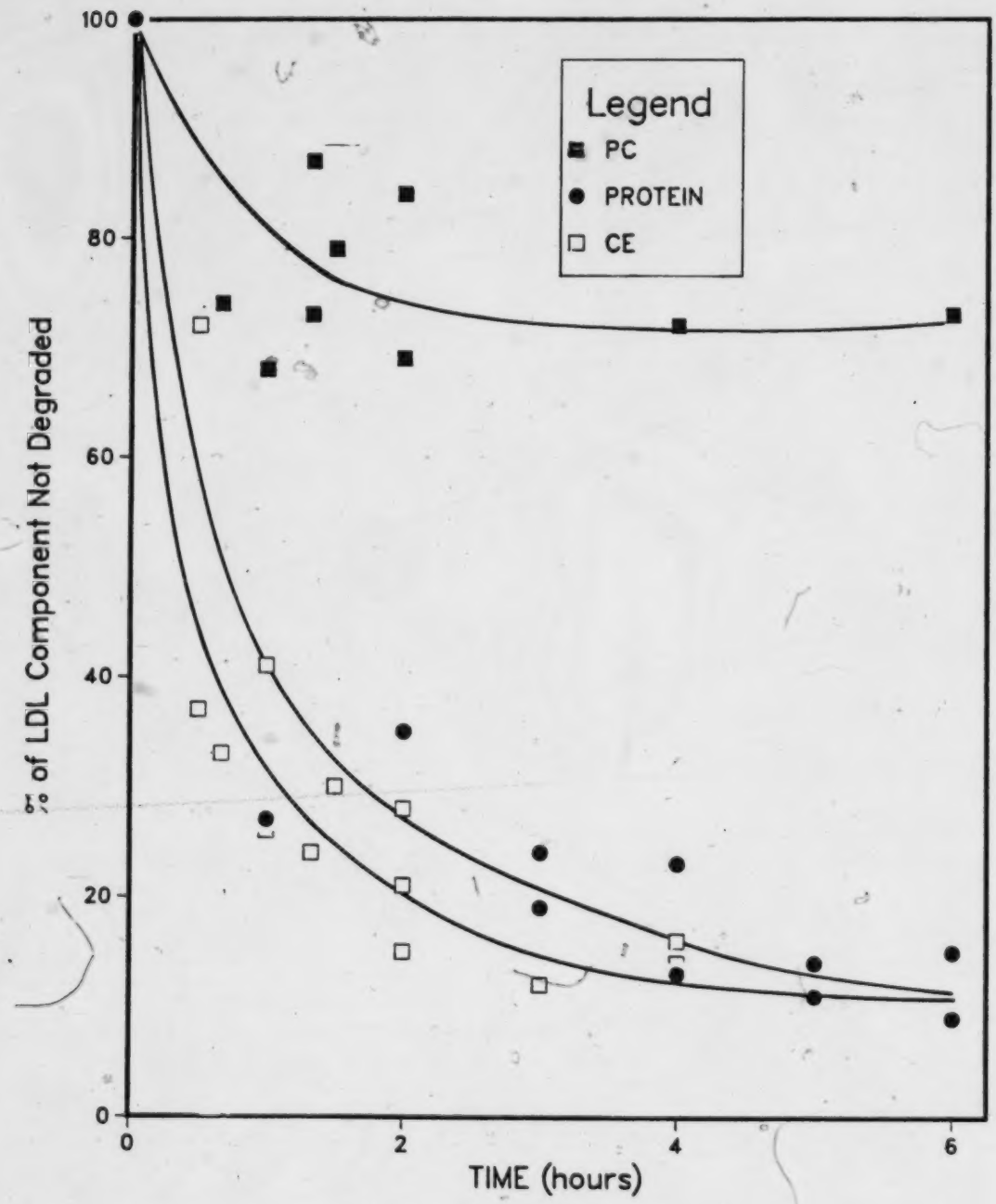
Figure 27. Time course of the conversion of the DPPC of r[^{3}H-DPPC]LDL to other PC species by normal fibroblasts. Incubation conditions were as described for Figure 26. Lipid extracts were chromatographed by polar tlc and the content of radioactivity at the origin and in the PC spot were determined as described under Materials and Methods. Some samples were treated with osmium tetroxide (+ osmium) before tlc. Each value is the mean of duplicate incubations which did not vary by more than 20%.



3.4 Summary of the Degradation of LDL Components

A summary of the degradation of the various LDL components by human fibroblast cells is shown in Figure 28. The data are compiled from a variety of experiments with single and double-labelled reconstituted LDL particles. In agreement with other studies (Krieger *et al.*, 1978; Goldstein and Brown, 1974), it has been demonstrated that, following the uptake and delivery of LDL to lysosomes, both the protein and cholesteryl ester components are rapidly hydrolyzed. In contrast, the results of experiments in which fibroblasts were incubated with reconstituted LDL particles containing different phosphatidylcholines suggest that LDL-associated PC molecules escape lysosomal hydrolysis and remain substantially ($>$ 75%) intact.

Figure 28. Summary of the cellular degradation of the various components of LDL.



4. DISCUSSION

The factors which determine the movement of lipids between cultured cells and serum are complex. It has become increasingly appreciated that extracellular sources of lipid have profound effects on cellular lipid composition and metabolism. More recently, attention has been focussed not only on understanding how lipids move in and out of cells, but also on how they are transported between the various membranes within cells (Voelker, 1985). In addition, although the compositional heterogeneity of the membrane lipids of different cell organelles has become well established, the question of how such diversity of structure is generated and maintained has yet to be answered. In the present studies, two different types of lipid movements between cultured human skin fibroblasts and extracellular lipid sources have been investigated.

In the first series of experiments, the effects of non-receptor mediated movements of cholesterol to or from normal and FH fibroblasts were determined.

In order to examine the effects of passive exchange movements of cholesterol on overall cholesterol homeostasis in the absence of any protein mediated pathways, fibroblasts were incubated in serum-free medium containing lipid vesicles of various cholesterol/phospholipid (C/P) ratios. The absence of serum eliminated any complicating sterol fluxes, either from the LDL receptor pathway or due to efflux to other serum components. It also avoided the possible effects of LDL on cellular phospholipid metabolism (Poznansky *et al.*, submitted), or effects of other serum factors on cell growth. Sonicated lipid vesicles were used to alter cell cholesterol content because they are well characterized, relatively easy to prepare containing radioactive tracers, and can be used to either load or deplete membrane cholesterol.

There are three key activities which are involved in the regulation of cultured fibroblast cholesterol levels: cholesterol synthesis activity, cholesterol esterification activity, and LDL receptor number. Each of these activities responds to cholesterol delivered to the cell via the receptor-mediated endocytosis and degradation of the entire LDL particle (Brown and Goldstein, 1975; Brown *et al.*, 1974; Goldstein *et al.*, 1974). The results of the present studies indicate that these activities are also responsive to cholesterol that enters the cellular pool via

passive exchange at the plasma membrane. Incubation of normal fibroblasts with either LDL or cholesterol rich vesicles resulted in similar changes in cell cholesterol content, cholesterol synthesis and esterification activities, and LDL receptor number. However, although both LDL and the vesicles increased cell cholesterol after 6 hours of incubation, there were differences in the time course of other effects. LDL exerted clear effects on synthesis, esterification, and binding within 6 hours. The effects of the C/P = 2 vesicles were delayed compared to those of LDL, with clear changes in the various activities not evident until after 24 hours.

The mechanism by which cholesterol exchanged into the cell at the plasma membrane is able to participate in the regulation of intracellular cholesterol metabolism is unknown. Although by all accounts cholesterol appears to move quite rapidly between (Green, 1983), within (Bell, 1984), and across (Backer and Dawidowicz, 1981) a variety of model and biological membranes, it is clear that there is an uneven distribution of cholesterol within the cell. The plasma membrane, lysosomal membranes, and probably Golgi apparatus membranes are sterol rich (Lange and Ramos, 1983; Thompson, 1980). Other intracellular membranes, particularly those of the endoplasmic reticulum, are believed to be relatively sterol poor and are typically isolated as microsomes with C/P ratios of approximately 0.1 to 0.2 (Hashimoto *et al.*, 1983). This is an interesting result in view of the fact that it is the endoplasmic reticulum which is the site of cholesterol synthesis. A possible explanation for this observation is provided by experiments from a number of laboratories which suggest that the translocation of newly synthesized sterol from the endoplasmic reticulum to the plasma membrane is a relatively rapid process, occurring with a half-time of about 10 to 30 minutes (DeGrella and Simoni, 1982; Mills *et al.*,., 1984; Lange and Matthies, 1984). On the other hand, it appears that plasma membrane cholesterol does not readily equilibrate with intracellular membranes (Poznansky and Czekanski, 1982; Shireman and Remsen, 1982; Slotte and Lundberg, 1983a; Kaplan *et al.*, 1984).

Since it can be estimated that cultured cells such as fibroblasts internalize the equivalent of their surface area approximately every hour (Steinman *et al.*, 1983; Farquhar, 1983), it is

unclear how this separation of plasma membrane and intracellular cholesterol pools is maintained. Bretscher (1976, 1984) has proposed that coated pits, which are the sites of internalization of cell plasma membrane, act as "molecular filters" which may exclude various membrane components, including cholesterol. Evidence for this hypothesis comes from freeze-fracture electron microscopy which showed the absence of filipin-sterol complexes in the coated pits of cell plasma membranes (Montesano *et al.*, 1979; but for a contrary view see McGookey *et al.*, 1983, and Steer *et al.*, 1984). Another possibility is that plasma membrane cholesterol may be internalized into the cell but then at some point become segregated from the endocytic pathway and recycled to the cell surface, as has been demonstrated for several cell-surface receptors, including the LDL receptor (reviewed by Brown *et al.*, 1983). The results presented here are consistent with either of these hypotheses; it may well be that cholesterol delivered directly to the cell interior via the endocytosis and degradation of LDL may gain access to any intracellular regulatory pool(s) more rapidly than cholesterol exchanged into the cell at the plasma membrane. This would explain why cholesterol delivered to the cell by exchange from vesicles requires a longer period of time than does LDL cholesterol to effect changes in intracellular cholesterol metabolism. Under these conditions, it may be that the movement of cholesterol from the plasma membrane to the cell interior is rate-limiting. This may have also been the case when LDL was incubated with FH fibroblasts. Under these conditions, it would be expected that cholesterol would enter the cell via non-receptor mediated exchange. The changes in intracellular cholesterol metabolism (particularly esterification) observed for FH cells in response to LDL were delayed and resembled qualitatively the changes observed when normal fibroblasts were incubated with C/P = 2 vesicles. This again suggests that it may be the movement of cholesterol from the cell surface to the endoplasmic reticulum which delays the response observed.

Conversely, the results from experiments involving cellular cholesterol efflux to egg PC vesicles imply that, in this case, the efflux from the plasma membrane is the rate-limiting step since the changes in both cell cholesterol content and intracellular metabolism occur together

after 24 hours of incubation. The fact that the cholesterol efflux to egg PC vesicles appears to be a slower process than the influx from the cholesterol rich (C/P = 2) vesicles (24 hours versus 6 hours) is not unexpected, considering that the cells were preincubated in LPDS which had already reduced the cell cholesterol content.

The physiological significance of these kinds of passive, non-receptor mediated movements of cholesterol is not known. It is clear that under cell culture conditions, substantial amounts of cholesterol can be delivered to fibroblasts by exchange. For normal fibroblasts incubated in medium containing 10% LPDS for 24 hours (maximal LDL receptor activity induced) and then incubated with 20 μG/ml LDL, typically an uptake of LDL protein in the order of 0.5 to 1 μg/mg cell protein/hr was observed. This represents a flux of cholesterol into the cell via the LDL receptor pathway of about 1.5 μg/mg protein/hr. The flux of sterol from cholesterol rich (C/P = 2) vesicles to fibroblasts was observed to be about 1 μg/mg protein/hr (cf. Table 5). Therefore, even with full up-regulation of the LDL receptor pathway, approximately equal amounts of cholesterol can enter the cell via receptor mediated and non-receptor mediated routes. Under more physiological conditions *in vivo*, or for cells cultured in whole serum *in vitro*, the LDL receptor activity will be reduced (typically to 5-10% of maximal activity) and under these circumstances the movement of cholesterol into cells will have an increasingly important non-receptor mediated component.

It is unclear how cholesterol introduced into fibroblasts via exchange is transferred to the cell interior or if it can be used for new membrane synthesis. Experiments by Goldstein *et al.* (1979) have indicated that cholesterol delivered to cultured fibroblasts via exchange from LDL or HDL cannot support the prolonged growth of these cells, perhaps indicating that net amounts of cholesterol had not been delivered. Yet it is known that such a mechanism is at least possible; the microorganism *mycoplasma* has been shown to derive its membrane cholesterol via passive equilibration with the cholesterol of serum lipoproteins (Razin and Rottem, 1978). In addition, it has been demonstrated that mouse splenic lymphocytes, which are unresponsive to mitogenic stimuli when cultured in the presence of cholesterol synthesis inhibitors, do respond

and divide when their membranes have been enriched with cholesterol obtained via exchange from synthetic lipid vesicles (Ip *et al.*, 1980).

The role of cholesterol efflux from tissues occurring via exchange from the cell surface (most likely to an HDL subfraction *in vivo*) may also be very significant in determining cellular and serum cholesterol homeostasis. As shown in the experiments demonstrating cholesterol efflux to egg PC vesicles, cholesterol exchange from the cell surface will increase LDL receptor activity and *in vivo* would be expected to increase the peripheral catabolism of LDL, perhaps resulting in reduced serum LDL levels.

The second type of lipid movement investigated in this series of experiments was the receptor mediated uptake of LDL-associated PC by cultured fibroblasts. A procedure for the incorporation of different radiolabelled PC molecules into a recombinant LDL particle was devised, based on a modification of the procedure of Krieger *et al.* (1978). The reconstituted LDL particles obtained appeared to be very similar in composition and biological activity to those described in the original method. The rLDLs had similar contents of CE and phospholipid as native LDL, but lacked free cholesterol and triacylglycerols. Apparently a substantial amount of the radiolabelled PC was incorporated by an exchange process rather than by net addition of phospholipid.

It was possible to selectively label the recombinant LDL particles in the cholesteryl ester, phosphatidylcholine, and apolipoprotein B components and selectively follow their uptake and metabolism by normal and receptor deficient fibroblasts. There are several lines of evidence which demonstrate that the various rLDLs were recognized and catabolized via the LDL receptor pathway described by Goldstein and Brown (1977):

1. rLDL competed against the binding of ^{125}I-LDL to normal fibroblasts almost as effectively as native LDL;
2. rLDL was not taken up by FH fibroblasts, which lack functional LDL receptors;
3. cellular uptake of rLDL by normal fibroblasts cultured in whole serum was $< 10\%$ of the uptake by cells exposed to 10% LDPS;

4. the apoprotein moiety of rLDL was degraded intracellularly to amino acids which were released into the culture medium;
5. the cholesteryl linoleate component of rLDL was rapidly hydrolyzed by cells to free cholesterol and fatty acid by a process which was inhibited by the lysosomal inhibitor chloroquine;
6. the cholesterol liberated by the cellular hydrolysis of the rLDL CE down regulated cellular cholesterol synthesis and stimulated cholesterol esterification.

Despite these results indicating that rLDLs were delivered via the LDL receptor pathway to the fibroblast lysosome for hydrolysis, it was consistently observed that LDL-associated PC remained substantially intact during the time course of the incubation with cells (typically 6 hours). Apparently, therefore, although the PC is internalized into the cell (as evidenced by the lack of PC uptake into FH cells or normal cells grown in the presence of whole serum), it is somehow resistant to lysosomal hydrolysis.

That this apparent resistance to catabolism is not due to a breakdown/reutilization cycle of PC components was demonstrated by the result that at least three quarters of internalized rLDL-associated DPPC remained intact and had not been converted to another PC species following 7 hours of incubation.

Evidence that at least a portion of the LDL-associated PC was delivered to the lysosomes for hydrolysis was provided by experiments in which the incubation of rLDL containing PC radiolabelled in the choline head group with normal fibroblasts resulted in the conversion of 5-8% of the radioactivity in the cell into a water soluble form. This conversion was inhibited in the presence of chloroquine and likely represents lysosomal hydrolysis of a portion of the internalized PC.

The mechanism by which LDL-associated PC escapes lysosomal hydrolysis is not certain but a strong possibility is that the phospholipid in the outer shell of LDL is available for exchange with endogenous cellular lipid somewhere along the endocytic pathway before actually arriving at the lysosome. There are several prelysosomal or endosomal compartments which

have been described, but the movements of the lipids which make up the membranes of these compartments have not been investigated. Little is known either of the rates of turnover of these lipids.

In general, the turnover of cellular phospholipids appears to be an exceedingly complex phenomenon. Estimates for the turnover of different phospholipids in different tissues and cell types range from a few hours to several days (Van den Bosch, 1980). And even within a given particular class of phospholipid, different molecular species appear to turn over at varying rates. For cells grown in culture, the situation is further complicated by factors such as the confluency of the cultures (Cunningham, 1972) and the possible stimulation of lipid turnover by hormones or other factors such as platelet derived growth factor (Leslie, 1982).

The role of degradative processes in determining overall phospholipid turnover is also somewhat unclear. Although most phospholipids are synthesized in one location, their degradation may take place at various sites within the cell, and it is possible that the heterogeneity of rates of turnover is a result of the various lipase activities present in different locations within the cell. For example, D'Sousa *et al.* (1983) have demonstrated that the turnover of exogenous PC introduced into cultured cells by exchange at the plasma membrane has a slower turnover than PC synthesized *de novo*.

It has been challenging to study the degradation of phospholipid in a systematic manner because it is difficult to selectively label a single pool of phospholipid which is all degraded within the same cellular compartment. Therefore, one approach to the study of phospholipid degradation by lysosomes has been to isolate the various lysosomal phospholipase activities and to study their characteristics *in vitro*.

There are difficulties in extrapolating from *in vitro* assays of phospholipase activity to their normal physiological function (Van den Bosch and Aarsman, 1979). Substrates may be presented to the enzyme in a variety of physical states in part determined by the characteristics of the phospholipid but influenced also by the method employed to disperse the substrate (detergents, bile salts, other lipids, etc.). This could account for some of the differences

observed in substrate specificity for different preparations of enzyme.

Another potential method for the assay of intracellular lysosomal phospholipases is to use the reconstituted LDL described in these studies. Although much of the lipid appears to escape hydrolysis at least a portion of the PC appears to reach the lysosomes and is catabolized. With reconstituted particles containing PC molecules double-labelled in the acyl chains or head groups, it may be possible to determine the *in vivo* rates of, for example, lysosomal phospholipase A_1 versus A_2 or phospholipase C activities. As well, the same method could be used to study the other major LDL phospholipid, sphingomyelin (SM), which is enriched in cell plasma membrane and appears to be turned over by a process which may involve lysosomal sphingomyelinase (Lastennet *et al.*, 1975; Sutrina and Chen, 1984).

Finally, the fact that PC appears to be taken up by cells but not hydrolyzed has some interesting implications in the regulation of cellular PC synthesis. We have shown (Poznansky *et al.*, submitted) that LDL will down regulate endogenous PC synthesis in human fibroblasts and lymphocytes. This phenomenon has also been previously reported for SM (Verdery and Theolis, 1982). The mechanism by which this down regulation occurs is not known, but further investigation of the catabolism and intracellular localization of LDL-associated PC may provide an insight into this process.

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